PLASMA MYELOPEROXIDASE LEVELS IN TYPE-2 DIABETES MELLITUS

DISSERTATION SUBMITTED FOR

M.D. DEGREE

BIOCHEMISTRY – BRANCH XIII

THE TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

COIMBATORE

APRIL – 2015

CERTIFICATE

This is to certify that the dissertation titled **"Plasma Myeloperoxidase levels in Type-2 Diabetes Mellitus"** submitted by **Dr.R.Sujatha** is an original work done by her at PSG Institute of Medical Sciences and Research, Coimbatore. This work was done under the guidance of Dr.B.Gayathri, Associate Professor, Department of Biochemistry, PSG Institute of Medical Sciences and Research.

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I solemnly declare that this dissertation "**Plasma myeloperoxidase levels in type-2 diabetes mellitus"** was written by me in the Department of Biochemistry, PSG Institute of Medical sciences and Research, Coimbatore, under the guidance of **Dr. B. Gayathri**, Associate Professor, Department of Biochemistry, PSG Institute of Medical Sciences and Research, Coimbatore.

This dissertation is submitted to the Tamil Nadu Dr. M. G. R Medical University, Chennai in partial fulfilment of the university regulations for the degree of M.D Biochemistry – Branch XIII examinations to be held in April 2015.

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Dr. R. SUJATHA

ABBREVIATIONS

ADA	- American Diabetes Association
AGE	- Advanced Glycation End products
BMI	- Body Mass Index
CETP	- Cholesterol Ester Transfer Protein
CVD	- Cardiovascular Disease
DM	- Diabetes Mellitus
FFA	- Free Fatty Acids
Hb	- Hemoglobin
HbA_1c	- Glycated hemoglobin
HDL	- High Density Lipoprotein
IFG	- Impaired Fasting Glucose
IGT	- Impaired Glucose Tolerance
LDL	- Low Density Lipoprotein
LPL	- Lipoprotein Lipase
MODY	-Maturity Onset Diabetes of the Young
MPO	- Myeloperoxidase
NEFA	- Non-Esterified Fatty Acids
NADH	-Nicotinamide Adenine Dinucleotide Hydrogen
NADPH	- Nicotinamide Adenine Dinucleotide phosphate Hydrogen

PAI-1 - Plasminogen Activator Inhibitor-1

PK-C - Protein Kinase-C

VLDL - Very Low Density Lipoprotein

WHO - World Health Organization

PSG Institute of Medical Sciences & Research

Institutional Human Ethics Committee

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November 22, 2012

To
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Ref.: Study titled: Plasma myeloperoxidase levels in type 2 diabetes mellitus

Sub.: Ethics Committee Approval

Dear Dr Sujatha,

In the Institutional Human Ethics Committee meeting held on 26.10.2012, at the College Council Room, PSG IMS&R, between 2.00 pm and 5.00 pm, the documents related to the above proposal were reviewed and discussed.

The following documents were received for review:

1. Duly filled application form
2. Informed Consent forms in English and Tamil
3. Data Collection Tool
4. Permission letter from the Medical Director
5. CV

The members who attended the meeting at which your study proposal was discussed are as follows:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
2	Mrs. R. Geetha	+ 2	Lay person	Female	No	Yes
3	Mr Gowpathy Velappan	BA., BL	Legal Advisor	Male	No	Yes
4	Mrs G Malarvizhi	M Sc	Nursing	Female	Yes	Yes
5	Mr. R. Nandakumar (Vice-Chairperson, IHEC)	BA., BL	Legal Expert	Male	No	Yes
6	Dr. G. Rajendiran	DM	Clinician (Cardiology)	Male	Yes	Yes
7	Dr. V. Ramamurthy	Ph D	Biotechnology	Male	Yes	Yes
8	Dr. M. Ramanathan	M Pharm, Ph D	Non-Medical (Pharmacy)	Male	Yes	Yes
9	Dr. P. Sathyan (Chairperson, IHEC)	DO, DNB	Clinician (Opthalmology)	Male	No	Yes

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10	Dr. Seetha Panicker	MD	Clinician (Obstetrics & Gyanaecology)	Female	Yes	Yes
11	Dr. S. Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
12	Dr. Y.S. Sivan	Ph D	Social Scientist (Sociology)	Male	Yes	Yes
13	Dr. Sudha Ramalingam (Alternate Member-Secretary, IHEC)	MD	Public Health, Epidemiology, Genetics, Ethicist	Female	Yes	Yes
14	Mrs. K. Uma Maheswari	M Sc, M Phil. B Ed	Botany	Female	No	Yes
15	Dr. D. Vijaya	M Sc, Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

After due consideration, the committee has decided to approve the above proposal.

The approval is valid for one year.

We request you to intimate the date of initiation of the study to IHEC, PSG IMS&R.

We hereby confirm that neither you nor any of your study team members have participated in the voting/ decision making procedure of the committee. The members of the committee who have participated in the voting/ decision making procedure of the committee do not have any conflict of interest in the referenced study.

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

PIs are required to send progress reports (in the form of an extended abstract with publications if any) to the IHEC every six months (and a month before expiry of approval date, if renewal of approval is being sought).

Request for renewal must be made at least a month ahead of the expiry of validity along with a copy of the progress report.

Yours truly,

22.11.12

Dr S Bhuvaneshwari
Member - Secretary

SECRETARY
PSG IMS & R
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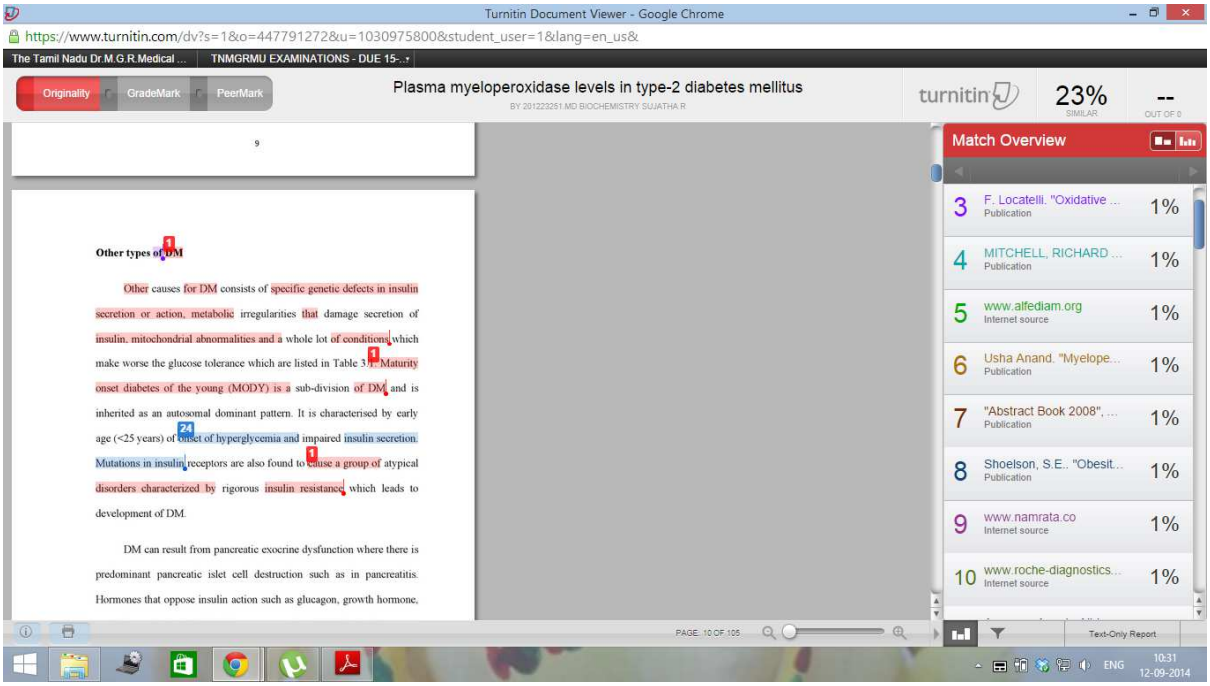
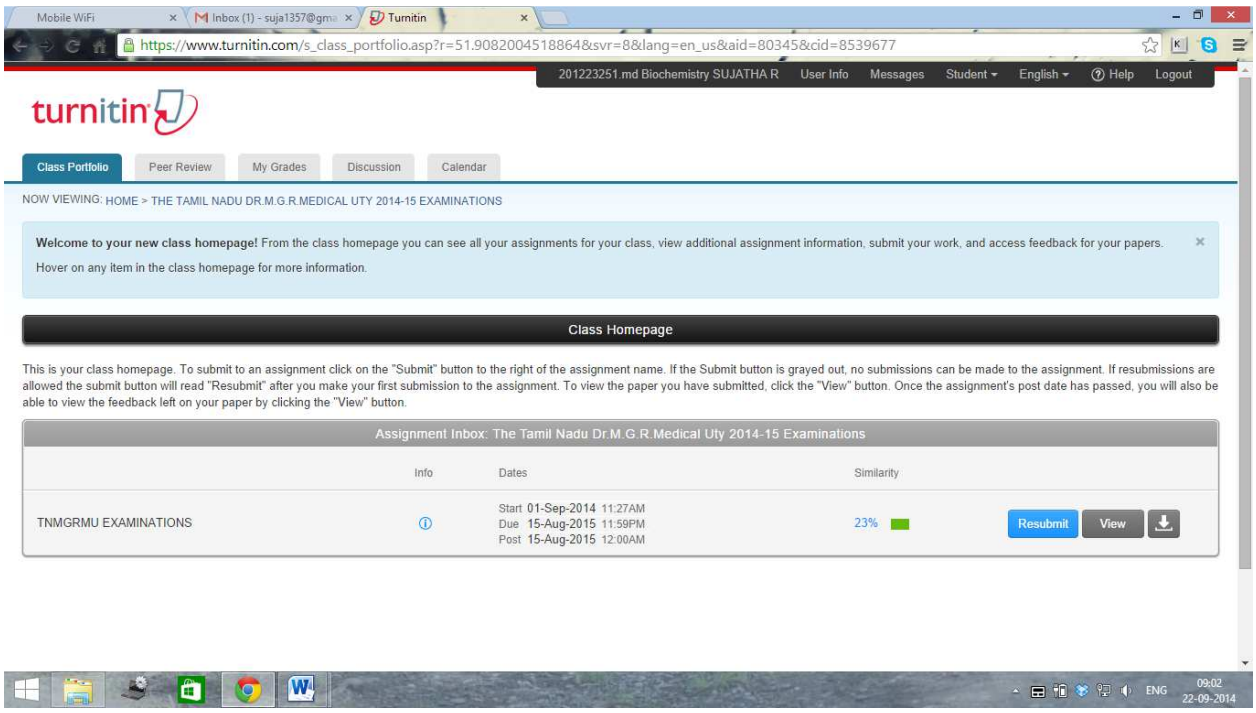
INSTITUTIONAL HUMAN ETHICS COMMITTEE

PLAGIARISM REPORT





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INTRODUCTION

Myeloperoxidase (MPO), an enzyme of the heme peroxidase superfamily in mammals is present within the azurophilic granules of leukocytes. It plays a major role in innate host defence by participating in the oxygen dependent killing of invading pathogens. MPO exhibits its anti-microbicidal property by catalyzing a unique reaction in which hydrogen peroxide reacts with chloride ions. The product formed by this reaction is the potent oxidant hypochlorous acid [1]. In addition, evidence shows that increased enzymatic activity of MPO is seen in many chronic inflammatory conditions. MPO is capable of generating reactive oxygen species that modify lipids and proteins which contributes to atherogenesis. Recent studies have shown that low density lipoprotein (LDL) on exposure to reactive nitrogen and halogen species by MPO gets converted into more atherogenic forms in the walls of the blood vessel [2]. Also MPO participates in the generation of dysfunctional high density lipoprotein (HDL) contributing to pro-inflammatory state which triggers atherosclerosis [3]. Apart from this, MPO consumes endothelial derived nitric oxide, thus reducing its bioavailability and impairing its functions such as vasodilatation and anti-inflammatory properties. Thus MPO is found to contribute to endothelial dysfunction, the major pathogenetic

mechanism in the process of atherosclerosis. Plasma MPO concentrations have been found to provide independent prognostic value to predict adverse outcomes in patient population with coronary artery disease [2].

Diabetes mellitus (DM) is a metabolic disorder, which occupies the seventh leading cause of death [4]. The burden caused by the morbidity and mortality in patients with type-2 DM is mainly driven by its vascular manifestations. The underlying pathology of vascular complications is centrally linked to amplified production of reactive oxygen species, specifically superoxide radicals and hydrogen peroxide (H_2O_2). Superoxide radicals generated from the uncoupling of electron transport chain is found to oxidize endothelial derived nitric oxide and thus lead to endothelial dysfunction. H_2O_2 derived from superoxide is the principal substrate for leukocyte peroxidases, in particular MPO. DM is associated with increased risk of premature vascular disease due to accelerated atherosclerosis. Inflammatory reactions take place in early phase of DM. Presence of pro-inflammatory state and endothelial dysfunction in DM are evidenced from previous studies [5].

The rationale of this study is to evaluate the plasma MPO activity in type-2 DM patients and to study their correlation with lipid profile and glycated haemoglobin (HbA_1c) levels of an individual.

AIMS AND OBJECTIVES

Aim:

The aim of the study was to estimate the plasma MPO activity in type-2 DM patients and non-diabetic controls.

Objectives:

1. To compare the plasma MPO levels in 2 groups of subjects namely non-diabetic controls and type-2 diabetic patients.
2. To study the correlation between plasma MPO activity and lipid profile in the study groups.
3. To study the correlation between plasma MPO activity and HbA_1c levels in the study population.

REVIEW OF LITERATURE

Introduction:

Diabetes mellitus is the most common non-communicable disease worldwide [6]. It describes a disorder of metabolism which is multifactorial in origin and characterised by chronic hyperglycemia. It is associated with metabolic disturbances related to carbohydrate, protein and fat which results from reduced insulin secretion or action or both [6].

History of DM:

Diabetes mellitus was first described in the Egyptian population. The term was first coined by Aertaeus, a Greek physician. The word diabetes meaning 'to pass through' in Greek was first used by Apollonius of Memphis in 250 BC. The word mellitus, the Latin word for honey was supplemented by Thomas Willis in 1600 to differentiate this disease from diabetes insipidus which was also linked with increased frequency of urination.

The disease was identified by the ancient Indian physicians Charaka and Susruta who classified it as Madhumeha or honey urine. They described the differences in the clinical presentation of patients with Madhumeha: some patients were thin, had severe polyuria, thirst, dehydration and few others were short, obese, ate excess food and led inactive life. The two groups were later classified as type-1 and type-2 DM respectively [7].

` The role for pancreas in diabetes was revealed by Joseph von Mering and Oskar Minkowski in 1889 who established that when pancreatectomy was done in dogs they developed all features of diabetes and died shortly afterwards [8]. Although diabetes has been known since pre-historic times and various effective treatments have been known in various parts of the world since the middle Ages, an effective treatment was developed after the Frederick Banting and Charles Best from Canada first used insulin in 1921 and 1922 [7].

Epidemiology of DM worldwide:

DM is the most common endocrine disorder worldwide. Type-2 DM accounts for more than 85% of diabetes cases in the world. In 2010, it was estimated that about 285 million people have diabetes in the world, of which 80% live in under developed countries [9]. The maximum number of people with diabetes is 76 million and seen in the Western Pacific region. The area with highest prevalence rate of 11.7% is in North America [9].The number of people with diabetes worldwide is expected to reach 438 million by 2030 [9].

Epidemiology of DM in India:

DM is reaching an exponential rate of epidemic proportions in India. The level of burden due to diabetes as a disease and its potential complications are massive. It poses significant healthcare burdens on the society. More than 62 million Indians are currently diagnosed with the condition [10]. It has been predicted that by 2030 DM may affect up to 79.4 million individuals in our country [11]. The increased number of diabetics in India is mainly due to a significant increase in the incidence of type-2 diabetes, caused by increased migration from rural to urban areas, which leads to changes in environmental and lifestyle [12].

India recently is exposed to an uncertain future with regard to the possible burden that diabetes forces upon the society. Many factors affect the prevalence of the disease in a country and identification of those factors is necessary to bring about changes in order to face challenges in the health care system.

Classification of DM:

An international expert committee set certain regulations in June 1997, and put forth new recommendations for the classification and diagnosis of DM [13]. It was recognised that the terms IDDM and NIDDM, although superficially pleasing, were often confusing as in patients with

insulin treated Type-2 DM. Hence the terms insulin-dependent DM (IDDM) and non-insulin-dependent DM (NIDDM) were made out dated. The classification was revisited by World Health Organisation (WHO) in 2006, but no further modifications were introduced.

The current classification of DM is on the basis of the pathophysiological process that progress to hyperglycemia. The two main categories are designated type-1 and type-2. Both types go ahead by a phase of abnormal phase of glucose equilibrium as the pathological processes progress. Type-1 DM results from total or near-total absence of insulin. Type-2 DM is a diverse group of disease characterized by unpredictable levels of insulin resistance, impairment in insulin secretion and high glucose production. Various genetic and metabolic irregularities in insulin action or secretion or both are also found to contribute to the common characteristic of hyperglycemia observed in Type-2 DM. Type-2 DM is ahead by an episode of abnormal glucose equilibrium state which is referred to as impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). These two intermediate conditions were removed from the formal classification of types of diabetes-but were retained as a risk state.

Table 3.1: Etiologic Classification of DM

I.	Type 1 diabetes (caused by islet cell destruction which usually leads to total insulin deficiency) May be due to either immune-etiology or idiopathic
II.	Type-2 diabetes (can vary from insulin resistance to insulin deficiency which mainly occurs as a result of defect in secretion)
III.	Other specific types:
A. Genetic defects of islet cell function characterized by mutations in:	
1. Hepatocyte nuclear transcription factor- 4 (MODY 1)	
2. Glucokinase (MODY 2)	
3. Hepatocyte nuclear transcription factor -1 (MODY 3)	
4. Insulin promoter factor-1 (MODY 4)	
5. Hepatocyte nuclear transcription factor -1 (MODY 5)	
6. NeuroD1 (MODY 6)	
7. DNA of mitochondria	
8. Subunits of ATP-sensitive potassium channel	
9. Pro-insulin or insulin	

B. Conditions which are caused by genetic defects in insulin action
1. Type A insulin resistance
2. Leprechaunism
3. Rabson-Mendenhall syndrome
4. Lipodystrophy syndromes
C. Diseases of the exocrine pancreas—pancreatitis, pancreatectomy, neoplasia, cystic fibrosis, hemochromatosis, fibrocalculous pancreatopathy, mutations in carboxyl ester lipase
D. Endocrinopathies—acromegaly, Cushing's syndrome, glucagonoma, pheochromocytoma, hyperthyroidism, somatostatinoma, aldosteronoma
E. Drug or chemical induced - glucocorticoids, Vacor (a rodenticide), pentamidine, nicotinic acid, diazoxide, β-adrenergic agonists, thiazides, hydantoins, asparaginase, α-interferon, protease inhibitors, anti-psychotics (atypical and others), epinephrine
F. Infections—congenital rubella, cytomegalovirus, coxsackie virus
G. Uncommon forms of immune-mediated diabetes—"stiff-person" syndrome, anti-insulin receptor antibodies.
H. Other genetic syndromes sometimes associated with diabetes—Wolfram's syndrome, Down's syndrome, Klinefelter's syndrome,

Turner’s syndrome, Friedreich's ataxia, Huntington's chorea, Laurence-Moon-Biedl syndrome, Myotonic dystrophy, Porphyria, Prader-Willi syndrome
IV. Gestational diabetes mellitus

Source: Adapted from American Diabetes Association, 2011.

Other types of DM

Other causes for DM consists of specific genetic defects in insulin secretion or action, metabolic irregularities that damage secretion of insulin, mitochondrial abnormalities and a whole lot of conditions which make worse the glucose tolerance which are listed in Table 3.1. Maturity onset diabetes of the young (MODY) is a sub-division of DM and is inherited as an autosomal dominant pattern. It is characterised by early age (<25 years) of onset of hyperglycemia and impaired insulin secretion. Mutations in insulin receptors are also found to cause a group of atypical disorders characterized by rigorous insulin resistance which leads to development of DM.

DM can result from pancreatic exocrine dysfunction where there is predominant pancreatic islet cell destruction such as in pancreatitis. Hormones that oppose insulin action such as glucagon, growth hormone, cortisol can also lead to DM. Thus, acromegaly and Cushing's disease can

also manifest as DM. Viral infections such as cox-sackie virus have a major concern in pancreatic islet cell destruction but are extremely rare causes of DM [14].

Gestational Diabetes Mellitus:

Glucose intolerance has also been found to develop during pregnancy. There is increased predisposition towards insulin resistance, particularly in the last trimester of pregnancy. The increased requirement of insulin during pregnancy leads to impairment in glucose tolerance. Gestational diabetes occurs in approximately 4% of pregnancies. Most women revert to normal glucose tolerance after delivery. However, they develop a 30-60% significant risk of developing DM later in life [14].

Impaired Glucose Tolerance (IGT):

IGT is diagnosed in people who have fasting blood glucose concentrations less than that required for diagnosis of DM, but have a plasma glucose response during the routine oral glucose tolerance test between that of a normal and diabetic state. An oral glucose tolerance test is required to diagnose a patient with this condition.

Impaired Fasting Glucose (IFG):

IFG is similar to IGT and is diagnosed by a fasting glucose levels above normal but below that needed for diagnosis of DM. It is a metabolic stage between normal glucose homeostasis and DM.

A diagnosis of IFG was defined as the fasting plasma glucose levels in the range of 100 mg/dL to 125 mg/dL.

The criterion for the diagnosis of IGT is 2-hour values following the oral glucose tolerance test between 140 mg/dL to 199 mg/dL.

Pre-diabetes refers to a state in which the patients are diagnosed with IFG or IGT conditions. These conditions designate the comparatively high threat for potential development of diabetes mellitus. They are viewed as risk factors for diabetes as well as cardiovascular disease. The natural history of both the conditions is variable. 25% of subjects with IFG or IGT can progress to apparent diabetes mellitus over 3-5 years. 50% remain in their intermediate glycemic status and about 25% regress to normal glucose tolerance over this period [15]. Pre-diabetic individuals are at high risk for cardiovascular disease, as a result of the metabolic abnormalities related to carbohydrate, protein and lipids along with the existence of known cardiovascular risk factors such as sub-normal levels of HDL cholesterol, increased levels of triglycerides and LDL cholesterol [16].

ADA 2010 Criteria for the diagnosis of type-2 diabetes mellitus:

1. Fasting plasma glucose ≥ 126 mg/dL.

   Fasting is defined as no caloric intake for at least 8 hours.

   OR

2. 2 hour plasma glucose ≥ 200 mg/dL during an oral glucose tolerance test.

   The test should be performed using a glucose load containing the equivalent of 75 grams anhydrous glucose dissolved in water, not recommended for routine clinical use.

   OR

3. In a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥ 200 mg/dL.

   Random is defined as without regard to time since the last meal.

   OR

4. HbA_1c ≥ 6.5%.

   The test should be performed in a laboratory using a method that is certified by the National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial assay.

*In the absence of unequivocal hyperglycemia and acute metabolic decompensation, these criteria should be confirmed by repeat testing on a different day [17].

Glycated haemoglobin (HbA_1c**):**

Glycated haemoglobin has been well-known as an index of blood glucose levels over a prolonged phase of time. It is an evaluation of risk for the evolution of complications in patients with DM.

Chemically, glycation is the addition of a sugar residue to amino groups of proteins which occurs spontaneously without the need for an enzyme. Human adult haemoglobin usually consists of HbA, HbA_2 and HbF which comprises of about 97%, 2.5% and 0.5% each of the total hemoglobin respectively HbA is made up of four polypeptide chains, two α and two β chains. HbA_1c is formed by reaction of glucose with the N-terminal valine residue of any one of the β-chains of HbA to form an unstable Schiff base. The Schiff base formation is reversible. This undergoes Amadori rearrangement to form a stable conjugate referred to as glycated haemoglobin. This reaction when it occurs between glucose and other proteins leads to the synthesis of advanced glycation end products (AGE) which contributes to the micro and macro vascular complications of DM. Glycation of collagen is found to alter its biological properties thereby increases cross linking and leads to the formation of brittle collagen. Adhesion of plasma proteins in the altered blood vessels leads to accumulation of LDL and consequent atherosclerosis.

After a broad review of recognized and rising epidemiological verification, an International Expert Committee, suggested to employ the HbA_1c test to establish a diagnosis of diabetes. The cut-off limit was set at a level of 6.5%. This test is considered valid if done using method qualified by the National Glycohemoglobin Standardization Program. The American Diabetes Association acknowledged this decision. The traceability of the method should be to the Diabetes Control and Complications Trial reference assay [18].

Major risk factors for Type-2 DM:

Table 3.2: Risk Factors for Type-2 DM

Family history of diabetes (parent or sibling with Type-2 DM)
Obesity (Body mass index (BMI $\geq$ 25 kg/m^2)
Physical inactivity
Race/ethnicity (African American, Latino, Native American, Asian American, Pacific Islander)
Previously identified with IFG, IGT or HbA_1c of 5.7-6.4%
History of Gestational diabetes or delivery of baby >4 kilograms
Hypertension (blood pressure $\geq$ 140/90 mmHg)
HDL cholesterol level < 35 mg/dL
Triglyceride level > 250 mg/dL

Polycystic ovary syndrome or acanthosis nigricans
History of cardio-vascular disease

Source: Adapted from American Diabetes Association, 2011.

Pathogenesis of Type-2 DM:

Type-2 DM is a heterogenous disorder, phenotypically, genotypically and pathogenetically. The most common form of type-2 DM is found to be the outcome of a grouping of genetic and acquired factors which negatively affect beta cell function and sensitivity of tissues to insulin [19]. For some years it was notorious whether impairment of the functions of beta cell or resistance to insulin action in peripheral tissues was the underlying fundamental element. The idea that insulin resistance was the basic defect had been traced back to the classic studies of Himsworth and Kerr [20]. Insulin resistance was found to result from the interplay of numerous genetic and environmental factors.

- ✓ **Insulin resistance:** Insulin resistance is defined as lack of ability of insulin to produce its typical biological functions at effective circulating levels in normal subjects [21]. It is found in non-diabetic individuals who are obese and in patients with Type-2 DM. It is usually attributed to defective insulin action. There is a broad scientific continuum of insulin resistance that varies from euglycemia with a remarkable increase in insulin secretion by the

pancreatic islet cells to hyperglycemia in spite of increased doses of exogenous insulin.

- ✓ **Loss of beta-cell function:** The impairment of beta cell function is associated with the insulin resistance mediated increased beta-cell demand that is required for developing fasting hyperglycemia. The major defect is loss of secretion of insulin which is induced by glucose termed selective glucose insensitivity. Excess increase in blood glucose levels renders the beta cells insensitive to glucose. The extent of beta cell dysfunction is found to be associated with both glucose concentration and duration of hyperglycemia. The normal pulsatile secretion of insulin is also found to be disturbed.
- ✓ **Diabetogenes:** Genetic factors also contribute to the development of diabetes. Type-2 diabetes is a polygenic disorder which means that multiple genetic polymorphisms must be there with or without acquired abnormalities in order to cause diabetes. Genes that affect beta cell apoptosis, beta cell regeneration, sensing glucose levels, ion channels, energy transduction, microtubules or microfilaments, metabolism of glucose and other islet proteins mandatory for the synthesis, binding, progress and discharge of secretory granules [22]. Until recently, only a small number of polymorphisms have been identified as risk factors: One involves amino acid genetic variations in the peroxisome proliferator activated receptor gamma

which is expressed in target tissues of insulin action and beta cells. Second involves the gene encoding a cysteine protease namely calpain-10, which modulates insulin release as well as insulin actions on skeletal muscle and adipose tissue [23]. Multiple genetic influences interact with exogenous factors such as environmental agents to produce the diabetic state of the individual. However, despite extensive efforts, the gene or genes causing the frequent form of Type-2 diabetes remains indefinite.

- ✓ **Environment:** Environmental factors ranging from dietary habits to level of physical activity are chief determinants in the development of Type-2 DM. Obesity is associated with insulin insensitivity and is the most significant predictive risk factor for development of Type-2 DM. It is found to be mediated by a variety of factors released from adipose tissue such as leptin, adiponectin and many others which adversely affect functions of beta cell. An inverse relationship exists between the level of physical activity and the prevalence of Type-2 DM. Exercise is thought to produce an increased sensitivity to insulin in skeletal muscle and adipose tissue.

Dyslipidemia in diabetes mellitus:

Insulin resistance in the perception of glucose metabolism leads to impairment in the suppression of endogenous glucose

production under basal and fasting conditions. It also leads to reduced peripheral uptake of glucose. Insulin suppresses the production of very low density lipoprotein (VLDL). Resistance to this action of insulin increases circulating serum triglycerides levels. Resistance to insulin action in the adipose tissue increases the discharge of non-esterified fatty acids (NEFA) both to the liver and skeletal muscle. This impairs the actions of insulin on glucose metabolism in these tissues. Evidence from previous studies [24] suggested that insulin normally suppresses the production of VLDL, especially VLDL -1 apo-B particles from the liver. This effect is brought about by decrease in availability of non-esterified fatty acids and also a direct effect of insulin on the liver cells where it inhibits the assembly and synthesis of VLDL particles [25]. In discrepancy to normal subjects, insulin fails to restrain VLDL apo-B production in those with Type-2 DM, though insulin profoundly lowers NEFA concentrations. Overproduction of VLDL and the defective insulin mediated inhibition of VLDL production are found to be one major causative mechanism for the increase in serum triglyceride concentrations in patients with insulin resistant Type-2 DM [26].

HDL levels are reduced in patients with insulin resistance accompanied by high serum triglycerides. There is increased

exchange of cholesteryl esters and triglycerides, between HDL and other lipoproteins that are rich in triglyceride, by cholesterol ester transfer protein (CETP) in conditions producing a hypertriglyceridemic state [27]. Finally the HDL particles are enriched with triglycerides which make them susceptible to the action of hepatic lipase. This clears HDL particles from the circulation at an increased rate. Further subnormal activity of lipoprotein lipase (LPL) leads to further fall in HDL cholesterol levels still further by decreasing the inter-conversion of HDL-3 to HDL-2 particles [27].

Elevated concentrations of VLDL particles in diabetic patients also increase the exchange of cholesterol ester and triglyceride between VLDL and LDL cholesterol particles mediated by CETP. This increases the triglyceride content of LDL particles which makes them more susceptible for the action of hepatic lipase [28]. This enzyme hydrolyses triglycerides present in the LDL particles thereby increasing their density. This sequence of events explains why Type-2 DM individuals have smaller and denser LDL particles than individuals without diabetes [29]. The small dense LDL particles are found to be highly atherogenic and provide a possible association between insulin resistance and cardiovascular disease [30].

Metabolic derangements in diabetes:

Carbohydrate metabolism in Type-2 DM:

People with Type-2 DM have fasting hyperglycemia and excessive variations in glucose levels following ingestion of a carbohydrate rich meal. In these patients insulin secretion is typically reduced and slow following food ingestion. Defects in insulin secretion are usually observed early in the evolution of Type-2 DM. In fact, variations in timing and amount of insulin secreted by the pancreatic beta cells have been reported in relatives of diabetic patients preceding the development of hyperglycemia.

Chronic hyperglycemia in combination with elevated free fatty acid levels impairs insulin secretion. Abnormalities in sensing glucose levels, processing of insulin or intracellular signalling modify insulin secretion. In addition, the mass of the pancreatic β-cell decrease with increase in the duration of diabetes. Alterations in β-cell morphology occur in most people with Type-2 DM with extensive intra-islet cell deposition of amylin commonly being observed [31].

Defects in insulin secretion and action are found to contribute to post-prandial rise in blood glucose levels. A delay in the rise of insulin concentration causes a delay in control of glucose production. This in turn

results in excessive glycemic variations. Also, a defect in insulin action leads to sustained hyperglycemia[32].

Glucose is an important regulator of its own metabolism. In the presence of insulin at basal levels, an increase in plasma glucose concentration stimulates uptake of glucose and suppresses its endogenous production. This ability of glucose to regulate its own metabolism is impaired in Type-2 DM. This is referred to as a defect in glucose effectiveness. The resulting excess increase in glucose is caused by impairment of glucose induced stimulation of glucose uptake [33].

Inhibition of glucagon secretion lowers both fasting and post-prandial blood glucose levels. When insulin secretion is intact, failure to suppress glucagon secretion has only minimal effect on glucose level regulation. In Type-2 DM in which insulin secretion is decreased and delayed glucagon causes marked hyperglycemia[34].

Amylin is a polypeptide made up of 37 amino acids. It is secreted by the pancreatic beta cell along with insulin in response to stimuli. Human studies have shown that plasma levels of amylin and insulin are found to rise and fall in parallel in both the fasting and fed states [35]. It has been implicated that excessive secretion of amylin may contribute to beta cell destruction in Type-2 DM.

In addition to defective insulin secretion, people with Type-2 DM frequently reveal defective insulin action. Several studies have shown that impairment of the suppression of glucose synthesis that is induce by insulin and stimulation of use of glucose in skeletal muscle and adipose tissue occurs in Type-2 DM [36, 37]. The severity of resistance to insulin action is influenced by several exogenous factors including exercise, obesity, diet and also genetic factors. Insulin resistance increases with increased severity of diabetes and improves by better glycemic control [38]. Defects in the capacity of insulin to regulate glucose metabolism in muscle and adipose tissue are evident in normoglycemic relatives of diabetic patients strongly favouring a genetic basis for insulin resistance.

Lipid metabolism in Type-2 DM:

Triglycerides are an important source of energy and storage form of lipids. They are mobilized as free fatty acids (FFA). Plasma FFA concentrations represent an equilibrium between their release and clearance. FFA are taken up and re-esterified in adipose tissue and hepatic cells. Some get oxidized in muscle (either cardiac or skeletal) and also in the liver. They are released from intravascular digestion of lipoproteins like chylomicrons which are rich in triglycerides and intra-adipocyte lipolysis of triglyceride stores. FFA concentrations are determined largely by its rate of access into circulation in fasting state. In

the post-prandial phase its rate of usage by both adipose and hepatic tissues also contributes to the FFA concentrations [39].

Hormone-sensitive lipase is the primary regulator in adipose tissue for the release of FFA. It is extremely sensitive to the hormonal actions of the principal regulator of lipolysis, which is insulin. Increased insulin secretion following an increase in glucose concentration after a meal inhibits lipolysis. This leads to a decreased in plasma FFA concentrations. It thus enhances insulin-dependent glucose clearance and insulin-induced suppression of endogenous glucose production [40].

In individuals with diabetes, the ability of insulin to suppress lipolysis is impaired, probably because of decreased sensitivity of hormone-sensitive lipase to insulin. The resulting increase in FFA tends to increase the blood glucose concentrations. Insulin also promotes FFA disposal by stimulating re-esterification in adipose tissue to form triglycerides. This is dependent on the availability of glycerol-3-phosphate derived from insulin induced uptake of glucose and intra-adipocyte glycolysis.

Circulating plasma triglyceride levels depends on the activity of LPL to deliver FFA to the adipose tissue. Insulin and glucose preferentially stimulate LPL in the adipose tissue and inhibit that present

in the muscle. It thus separates triglyceride-derived and lipoprotein-derived fatty acids away from muscle and into adipose tissue [41].

In Type-2 DM, insulin-induced activation of LPL in adipose tissue is delayed whereas there is activation of skeletal muscle LPL. FFA decrease glucose uptake in muscle by inhibiting glucose transport, glucose phosphorylation and muscle glycogen synthase [42]. Elevated FFA stimulates gluconeogenesis in liver and synthesis of triglycerides. Acute rise in FFA stimulates insulin secretion whereas chronic rise inhibits insulin secretion [43]. Thus, elevated FFA has been implicated in many of the metabolic abnormalities associated with Type-2 DM.

Protein metabolism in Type-2 DM:

Protein metabolism at any given time is generally regulated by substrate availability and the hormonal milieu. Insulin is found to have profound influence over protein metabolism. People with Type-2 DM have sufficient left over insulin secretion to limit protein breakdown and maintain lean body mass. On the other hand, whole body nitrogen flux, protein synthesis and breakdown are increased in poorly controlled diabetic patients. Improvement in glycemic control by treatment with either oral hypoglycemic agents or insulin restores these defects to normal.

Few studies have examined regional protein dynamics in Type-2 DM. 3-methylhistidine excretion, an index of myofibrillar protein breakdown has been found to be increased in individuals with poor control of their diabetic status when differentiated from healthy subjects[44]. Improvement in glycemic control decreased 3-methylhistidine excretion. Clotting factors such as tissue plasminogen activator are found in elevated concentrations in diabetic patients. In addition levels of plasminogen activator inhibitor-1 (PAI-1) are also increased in these individuals. This implies that the synthesis of certain proteins by the hepatocytes and endothelial cells is abnormal. This provides additional evidence of the increased threat of cardiovascular complications in diabetic individuals [45].

Complications of DM:

The majority of the burden due to the morbidities associated with type-2 DM is the result of the plethora of complications which ensues the development of the disease. The most common complications associated with it are classified as acute and chronic based on their mode of onset.

Acute complications:

1. **Diabetic ketoacidosis:**

   Ketosis is more common in type 1 DM. The normal level of ketone bodies in blood is less than 1 mg/dL and only traces get

excreted in urine. When rate of synthesis exceeds the ability of extrahepatic tissues to utilize them, they get accumulated in blood. The combination of hyperglycemia, glucosuria, ketonuria, and ketonemia is called diabetic ketoacidosis. Untreated DM is the most common cause for ketosis.

2. Hyperglycemic Hyperosmolar State:

The primary defects in this condition are a relative state of insulin deficiency and too little fluid intake. Deficiency state of insulin increases hepatic glucose production which occurs endogenously through glycogen breakdown and gluconeogenesis. It also impairs uptake of glucose in skeletal muscle. Hyperglycemia induces an osmotic diuretic state which leads to depletion of the intravascular fluid compartment. This is further exacerbated by inadequate fluid replacement. The absence of ketosis in this condition is not completely understood.

Chronic complications of DM:

The chronic complications of DM are known to affect various organ systems. They can be divided into vascular and non-vascular complications (Table 3.3).

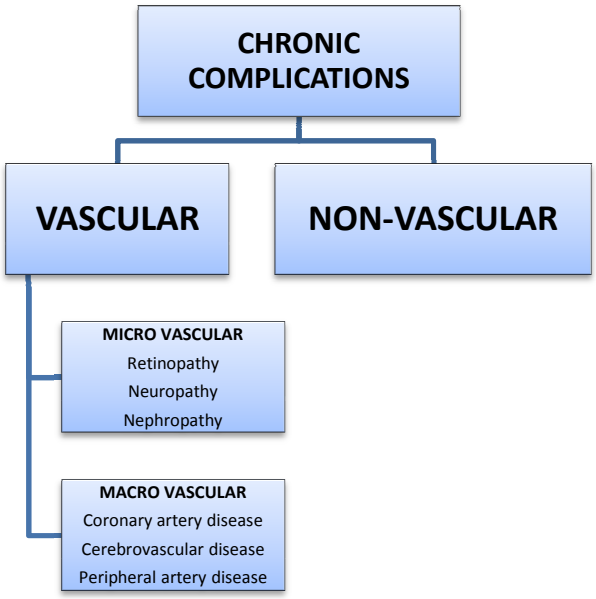


Table 3.3: Vascular Complications of DM

Microvascular
Eye disease
Retinopathy may be non-proliferative or proliferative
Macular edema
Neuropathy
Sensory and motor
Autonomic
Nephropathy
Macrovascular
Cardiovascular disease
Peripheral arterial disease
Cerebrovascular disease
Others
Gastrointestinal dysfunction such as gastroparesis, diarrhea
Genitourinary abnormalities
Dermatologic manifestations
Infections
Cataract
Glaucoma
Periodontal disease
Hearing loss

Source: Harrison's Principles of Internal Medicine 18th edition. Table 344-7.Page 2980. Alvin C Powers. Diabetes Mellitus

The risk of chronic complications increases with duration of hyperglycemia. Since Type-2 DM often is preceded by a long disease free period without any symptoms of hyperglycemia, many patients present with complications at the moment of diagnosis.

The micro-vascular complications of Type-2 DM are found to result from chronic hyperglycemia. Genetic susceptibility also has a predominant contribution to the pathogenesis of complications in diabetic patients. The role for chronic hyperglycemia in the development of macro-vascular complications is not much evident. However, coronary heart disease events and mortality are two to four times greater in patients with Type-2 DM. These events are also found to correlate with fasting, postprandial glucose levels in the blood and also with the HbA_1c. Other factors such as dyslipidemia and hypertension are also found to play important roles in development of vascular complications in diabetic patients [46].

Pathogenesis of vascular complications in DM:

Chronic hyperglycemia has been implicated as an important etiologic factor in the development of diabetic complications. Four theories were put-forth to explain the mechanisms by which it leads to such varied dysfunction of the cells and organs. One recent emerging

hypothesis suggests that hyperglycemia leads to epigenetic changes in the affected cells.

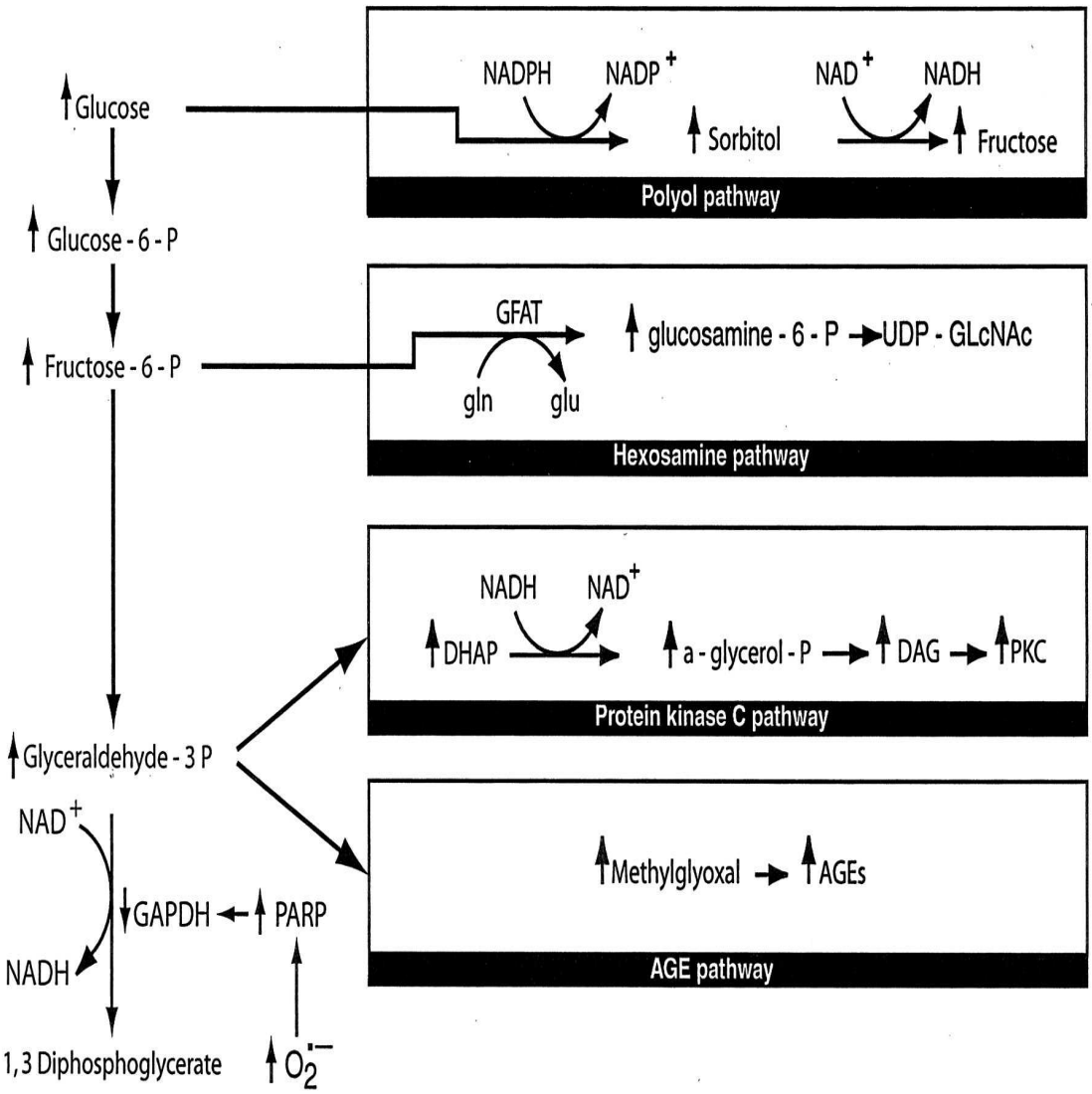
One theory is that chronic hyperglycemia can progress to the formation of AGE via the non-enzymatic protein glycation. This results from the condensation of glucose with amino groups present in proteins. AGE are found to cross-link extra cellular matrix proteins such as collagen. These glycated proteins are found to modify the composition and functions of the extracellular matrix and also exhibit a wide variety of effects on the vascular system such as decreased synthesis of nitric oxide, acceleration of atherosclerosis, glomerular dysfunction through modification of basement membrane proteins.

Second theory suggests that excess glucose that results from insulin deficiency is channelized into the sorbitol pathway. Normal metabolism of glucose inside the cells is usually by phosphorylation and subsequent glycolysis. When glucose levels are increased, it gets converted to sorbitol by the enzymatic action of aldose reductase. Increased concentration of sorbitol alters the redox potential, increases the cellular osmolality and also generates reactive oxygen species which in turn leads to cellular dysfunction.

The third hypothesis suggests that increased glucose levels in the blood increases the production of diacylglycerol. This activates the protein kinase-C (PK-C) signal transduction pathway. PK-C alters the transcription of genes for fibronectin, type IV collagen, contractile proteins and other proteins of the extracellular matrix present in endothelial cells and neurons.

A fourth presumption proposes that excess blood glucose leads to a major flux through the hexosamine pathway. This generates fructose-6-phosphate, a substrate for O-linked glycosylation and production of proteoglycans. This leads to glycosylation of proteins such as endothelial nitric oxide synthase or alters functions of certain proteins by regulating gene expression of transforming growth factor or PAI-1. Increased production of growth factors by one of the above proposed mechanisms is found to contribute to the vascular complications of DM.

Figure 3.1: Pathogenesis of vascular complications in DM



Source - Brownlee M. Pathobiology of diabetic complications. Nature 414: 2001; 813-820.

A possible unifying mechanism that leads to diabetic complications is that, raised blood glucose levels due to insulin deficiency or insulin resistance leads to increased creation of reactive oxygen species or

superoxide radicals in the mitochondria. These free radicals were found to be responsible for the deleterious effects on organ systems [47].

Cardiovascular morbidity in DM:

Diabetic patients are 2 to 4 times more likely to succumb to the drastic effects heart disease than non-diabetics. More than 70% of mortality in diabetic patients is attributed to cardiovascular disease (CVD) [48]. DM has been identified as a major risk factor for CVD by The American Heart Association. Patients with CVD and co-existing DM have a worse prognosis than that for non-diabetics. Further, multiple vessel involvement in CVD is more likely in individuals with DM.

The co-existence of hyperglycemia along with other major cardiovascular risk factors contributes to the increase in cardiovascular system related morbidity and mortality in diabetic individuals. The other major risk factors for macro-vascular disease in diabetic individuals include altered lipid profile, associated systemic hypertension, overweight and obesity, sedentary lifestyle and smoking habits. Further certain alterations in biochemical parameters more prevalent in diabetic patients also pose an additional risk to these individuals. These include micro and macroalbuminuria, elevation of serum creatinine levels and dysfunctional platelet activity. Insulin resistance itself is a well-

established risk factor for cardiovascular complications in individuals with or without DM. These individuals have raised levels of plasminogen activator inhibitors (especially PAI-1) and fibrinogen which enhances the coagulation process and impairs fibrinolysis thus favouring development of thrombus in the vasculature. The underlying mechanism for the increased cardiovascular morbidity in DM is accelerated atherosclerosis. The process of atherosclerosis starts from the formation of a fatty streak that gradually progresses into more advanced plaques. It terminates in complicated lesions of atherosclerosis, which through rupture and thrombus formation can lead to acute myocardial infarction.

Atherosclerosis:

It is characterized by lesions in the endothelial intimal layer called atheromas or atherosclerotic plaques that project into vessel wall lumen. An atheromatous plaque is a raised lesion made up of a central core of lipid which is made up of predominantly cholesterol and its esters covered by a white fibrous cap. Apart from obstructing blood flow by mechanical means, these plaques rupture and lead to distressing vessel thrombosis. It can also deteriorate the underlying media thus leading to formation of aneurysm [49]. Hyperlipidemia is one of the most important risk factor for atherosclerosis. The mechanism by which hyperlipidemia contributes to atherogenesis include thefollowing:

Stage 1: Formation of foam cells:

Chronic hyperlipidemia, especially increase in cholesterol levels can directly produce endothelial dysfunction by increasing free radical production locally. These reactive radicals can cause injury to the tissues and accelerates decay of nitric oxide thereby reducing its vasodilator activity.

Lipoproteins accumulate within the intima in hyperlipidemic conditions. They get oxidized through the action of oxygen free radicals generated locally by macrophages or endothelial cells. Oxidized LDL is ingested by macrophages through a scavenger receptor and accumulates in phagocytes which are further referred to as foam cells. Further, oxidation of LDL provokes the release of various growth factors, cytokines and chemokines by endothelial cells and macrophages. This increases further monocyte recruitment into atherosclerotic lesions. Finally, oxidized LDL is toxic to endothelial cells and smooth muscle cells and thus induces dysfunction of the endothelial cell. Accumulation of this modified LDL within macrophages in various stages of plaque formation reveals its importance in atherogenesis.

Stage 2: Progression of atherosclerosis: Smooth muscle cells containing lipid droplets are seen in the lesion. The condition is reversible in the early stages if lipid levels especially that of LDL is lowered.

However when lipid is accumulated, the lesion progresses and the arterial changes become irreversible.

Stage 3: Fibrous proliferation: There is a definite component of inflammation in atherosclerosis. Dysfunctional endothelial cells in the major arteries express a variety of adhesion molecules, particularly vascular cell adhesion molecule-1 which binds to monocytes and T-cells. These cells adhere to the endothelium; migrate into the intima under the control of locally produced chemokines. The transformation of macrophages into monocytes into macrophages leads to engulfment of lipoproteins, including oxidized LDL. Progressive accumulation of oxidized LDL promotes lesion development. Macrophage activation results in cytokine production which further increases adhesion of leukocyte and chemokine production. It also produces reactive oxygen species, aggravating LDL oxidation. T-lymphocytes that are recruited to the intima interact with macrophages and generates a chronic inflammatory state. However, activated T-cells elaborate inflammatory cytokines. This process stimulates macrophages, endothelial cells and smooth muscle cells proliferation mediated by various growth factors.

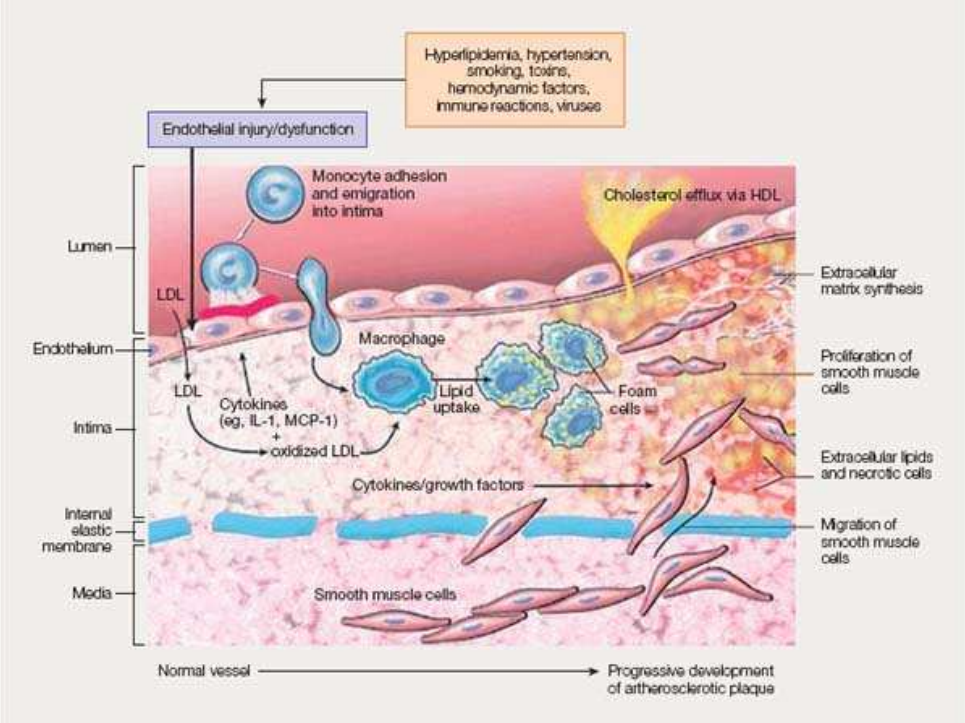
Stage 4: Advancing fibrous plaque: This leads to contraction of the blood vessel wall leading to more turbulent blood flow and increased tendency to clot formation [50].

Table 3.4: Major risk factors in development of atherosclerosis:

Modifiable	Non-Modifiable
Increased lipid levels in blood	Advancing age
Systemic hypertension	Males
Diabetes	Individuals with family history
Smoking – Tobacco	Genetic defects

Source: Kumar, Abbas, Fausto, *et al*. Robbins and Cotran Pathologic Basis of Disease, 8th Edition, Chapter 11, Blood Vessels, Richard N Mitchell, Frederick J Schoen, Elsevies- Saunders, Philadelphia, 2010: 497.

Figure 3.2: Sequence of cellular interactions in atherosclerosis



Source: Kumar, Abbas, Fausto, *et al*. Robbins and Cotran Pathologic Basis of Disease, 8th Edition, Chapter 11, Blood Vessels, Richard N Mitchell, Frederick J Schoen, Elsevies- Saunders, Philadelphia, 2010: 501.

Risk factors such as hyperlipidemia cause injury to the endothelial cells which results in sticking together of platelets and monocytes and release of growth factors which leads to smooth muscle cell migration and proliferation like platelet-derived growth factor. Macrophages and smooth muscle cells transform to foam cells that constitute the atheromatous plaques. It is derived from macrophages via the VLDL receptor. In conditions of increased cholesterol levels, the extracellular lipid is derived from accumulation in the vessel lumen and also from degenerating foam cells. Accumulation of cholesterol in the plaque reflects a disparity between the rate of its entry and exit from the circulation. HDL helps to clear the cholesterol resulting from these accumulations. Smooth muscle cells migrate to the intimal layer of the blood vessels, proliferate in it and produce extracellular matrix proteins, like collagen and proteoglycans [49].

Endothelial dysfunction in diabetes mellitus:

The increased occurrence of cardiovascular disease in diabetic patients is generally attributed to the unfavourable effects of hyperglycemia and oxidative stress. It has been revealed that pre-diabetic conditions, such as IFG and IGT pose an increased risk for development of cardiovascular disease [16]. Atherosclerosis which has been implicated in its pathogenesis is found to arise because of vascular endothelial cell

dysfunction due to the prevalence of any individual risk sources listed in Table 3.4.

Vascular endothelial cells are found to play a crucial role in maintaining cardiovascular environment. In addition to providing a physical obstacle between vessel wall and lumen, it also secretes numerous mediators which are found to control aggregation of platelets, coagulation process and vessel tone. They also produce various mediators which alternately mediate either vasoconstriction such as endothelin-1 and thromboxane A2 or vasodilatation such as nitric oxide, prostacyclins and endothelium-derived hyperpolarizing factor. In larger arteries nitric oxide is the major contributor to endothelium-dependent relaxation, whereas in the smaller resistance vessels contribution of endothelium-derived hyperpolarizing factor predominates [51].

Patho-physiology of endothelial dysfunction in Type-2 DM:

The main functions of endothelial cells are altered in Type-2 DM. There are a number of mechanisms for the commencement of impaired function of endothelium in type-2 diabetes described including hyperglycemia, formation of AGE and dyslipidemia.

Hyperglycemia:

Hyperglycemia contributes to endothelial dysfunction in a number of ways. Decreased activity of pentose phosphate pathway leads to limited availability of NADPH, a necessary cofactor for endothelial nitric oxide synthase which can cause reduced production of nitric oxide. Increased glucose transport across endothelial cell membranes leads to increased flux through polyol pathway. As a consequence glucose is reduced to sorbitol by aldose reductase, leading to a further depletion of NADPH. Sorbitol undergoes oxidation to fructose by sorbitol dehydrogenase enzyme. This reaction uses NAD^+, resulting in amplification of $NADH/NAD^+$ ratio, thereby leading to modification of the redox state of the cells. This results in oxidative stress, which promotes generation of superoxide anions which further quenches nitric oxide, reducing its bioavailability [52].

Formation of AGE:

Hyperglycemia increases the denovo synthesis of diacylglycerol by enhancing metabolism of glucose to diacylglycerol precursors through glycolysis. The diacylglycerol activates an important signal transducer namely PK-C. This leads to decrease in enzymatic activity of nitric oxide synthase, especially the endothelial isoform and an increase in the production of prostaglandin like substances by the endothelium [53]. PK-C

promotes generation of superoxide anions, which reacts with nitric oxide to produce peroxy-nitrite (ONOO-) which damages adjacent tissues. The amplified production of endothelin-1 due to the increase in glucose levels via activation of the β and δ isoforms of PK-C leads to its increased concentration in Type-2 DM [54]. The PK-C leads to the migration and proliferation of smooth muscle cells by increasing the production of growth factors by the endothelium,.

Hyperglycemia produces vascular dysfunction due to the interaction of AGE with their specific receptors on the endothelium. AGE quench the nitric oxide and increases the susceptibility of LDL to oxidation. The AGE and their receptors interact with each other which lead to a rise in thrombomodulin and activate the receptors for the numerous cytokines such as interleukin-1, tumour necrosis factor-α and growth factors leading to the migration and proliferation of smooth muscle cells. Other cell adhesion molecules are also increased related to hyperglycemia. This alters the vascular permeability which in turn favours the transmigration of mononuclear cells and the change of monocytes to macrophages which finally become foam cells of the atherosclerotic plaque [55].

Oxidative stress and diabetes mellitus:

Oxidative stress results from a disproportion between an augmented generation of oxidant compounds and inadequate anti-oxidant defence mechanisms which lead ultimately to tissue damage [56]. Generation of oxidant compounds is physiologically relevant as a defence mechanism against invading microorganisms, cancer cells and also in tissue healing and remodelling. Dysfunction in the oxidative mechanisms can contribute to cell injury and death.

Sources of oxidative stress:

The respiratory chain of mitochondria represents the most potent source of oxidants in the body. These oxidants exert deteriorating effects contributing to aging of the cells and tissue damage. The phagocytes in response to a suitable stimuli leads to the production of oxidants based on the stimulated production of reactive oxygen species via univalent reduction of oxygen at the molecular level. This leads to the activation of polymorphonuclear neutrophils, monocyte and macrophages to increase their oxygen consumption. This phenomenon is referred to as the respiratory burst.

The NADPH oxidase enzyme system remains attached to cellular membranes. It reduces molecular oxygen to the highly unstable superoxide anion ($O_2^{-}\cdot$), by its enzymatic action which finally gets

transformed into hydrogen peroxide. Both O_2^{-}•and hydrogen peroxide serve as important precursors for the synthesis of potent oxidants. Superoxide anion reacts with nitric oxide to produce highly reactive nitrogen species. Hydrogen peroxide, through Fenton's reaction reacts with intracellular iron to produce the hydroxyl radical [57]. Hydrogen peroxide oxidises ferrous iron (Fe^{2+}) to ferric form (Fe^{3+}), a hydroxyl radical along with a hydroxyl anion. This is followed by the reduction of the ferric form to ferrous, a superoxide radical and a proton by the same hydrogen peroxide.

(1) Fe^{2+} (Ferrous) + H_2O_2 +H^+ → Fe^{3+} (Ferric) + HO•(hydroxyl radical) + H_2O

(2) Fe^{3+} H_2O_2 + → Fe^{2+} + HOO• (Superoxide radical) + H^+ (proton)

These reactions have been concerned in degeneration of the lipid cell membrane, protein aggregation and DNA damage.

Hydrogen peroxide is the substrate for MPO to produce the chlorinated oxidants. In the presence of chlorine, MPO converts hydrogen peroxide into hypochlorous acid. This is a powerful compound capable of inducing the oxidation a number of molecules like lipids, proteoglycans and other membrane proteins or intracellular constituents, particularly the thiol group of membrane proteins. In addition, hypochlorous acid reacts with endogenous amines to form chloramine derivatives. The reactive

oxygen species are released together with pro-inflammatory cytokines, which again magnifies further oxidant generation.

Oxidative stress markers and antioxidants:

Oxidants are compounds which are highly reactive. They have a very short half-life of only a few seconds. Therefore, their estimation in-vivo is generally not possible. In contrast modification of lipids, proteins, carbohydrates and nucleic acids, by oxy-radicals, have biological activity ranging from hours to weeks. Thus they serve as ideal markers of oxidant stress [58].

Table 3.5: Markers of oxidative stress and anti-oxidants:

Oxidative stress markers	Anti-oxidants
1. Lipid peroxidation: Acrolein, Malondialdehyde, 4-hydroxynonenal, thiobarbituric acid reactive substances, Advanced lipid oxidation products, Oxidised LDL antibodies 2. Protein oxidation: Advanced protein oxidation products	1. Enzymatic Superoxide dismutase Catalase Glutathione peroxidase 2. Non-enzymatic Glutathione Vitamin C Vitamin E Ferritin Transferrin

3. Carbohydrate oxidation: Advanced glycation end products 4. Nucleic acid oxidation: 8-hydroxy-2' deoxyguanosine	Albumin

Role of oxidative stress in the pathogenesis of diabetes and diabetic complications:

The mechanisms of free-radical production in diabetes include glucose auto-oxidation, protein glycation, AGE formation and activation of polyol pathway. All these pathways ultimately lead to an oxidative stress in a number of tissues [59]. The lack of appropriate compensatory mechanisms from natural in-vivo antioxidant system produces an imbalance in the redox state of the cell. This leads to the activation of intracellular signalling pathways. The increased generation of reactive oxygen species causes damage of proteins, lipids and nucleic acids. Further the activation of signalling pathways known to regulate gene expression also leads to cellular damage [60].

Lipid peroxidation produced by the activity of free radicals play a major role in the development of diabetic complications. Lipids combine

with free radicals and undergo peroxidation. This leads to the formation of lipid peroxides which on decomposition form numerous by-products including malondialdehyde. This modified lipids after peroxidation decreases the membrane fluidity which leads to a dysfunction in the plasma membrane. It also changes the activity of membrane bound proteins. The lipid peroxidation products are harmful to every cell in the body and are related with a diversity of diseases including DM.

Inflammation in DM:

Inflammation has been proposed to be important supplementary causal factors in the progress of Type-2 DM. The central mechanism by which inflammation and stress are found to mediate insulin resistance is through the inhibition of insulin receptor signalling pathways.

The cells of the adipose tissue are an important source of storage of lipids and help in regulation of homeostatic environment for metabolism. It also regulates the functions of macrophages in inflammation. In obesity, there is inflammation of adipose tissue. This leads to an infiltration of adipose tissue by macrophages and as a result of adipocytes produce their own inflammatory cytokines. This inflammatory response of the adipose tissue is a vital step in the progression of peripheral insulin resistance.

The numerous pathways of inflammation are found to be initiated by extracellular mediators or by intracellular stress, mainly oxidative

stress which leads to over-production of reactive oxygen species in the mitochondria. Signals from all of these mediators converge on inflammatory signalling pathways, which lead to the production of further mediators of inflammation through regulation of transcription regulation as well as to the direct inhibition of insulin signalling [61].

Inflammatory cytokines:

Interleukin-6:

The pro-inflammatory cytokine interleukin-6 was among the first to be implicated a as predictor of insulin resistance and cardiovascular disease. There is an increased circulating level of interleukin-6 in individuals with Type-2 DM. The production of interleukin-6 by the abdominal adipose tissue is much higher than that from the subcutaneous adipose tissue. This points towards the fact that interleukin-6 may be one of the factors that make central adiposity a high risk factor for the development of insulin resistance [62].

Interleukin-10:

It is an anti-inflammatory cytokine. The decrease of its synthesis is found to be associated with the development of Type-2 DM. The immune cells in the adipose tissue are found to produce this anti-inflammatory factor. It acts on adipocytes to improve signalling pathways of insulin action and also decreasing further macrophage recruitment [63].

Tumour Necrosis Factor-α:

It is a cytokine secreted both by immune cells and adipocytes. It has been found to play a role in the development of insulin resistance [64].

Resistin:

Resistin is an inflammatory cytokine produced by the cells in the adipose tissue and immune cells. Its expression is found to be suppressed by thiazolidinediones and is up-regulated by pro-inflammatory cytokines and bacterially derived lipopolysaccharide [65]. It stimulates intracellular signalling through NF-kB activation, which in turn promotes the synthesis of other pro-inflammatory cytokines and surface adhesion molecules [66].

Monocyte chemoattractant protein-1:

It is a chemoattractant protein. Its main role is to recruit the cells of the immune system to inflammatory sites. Increased levels of this protein are implicated in onset of several diseases which are represented by the infiltration of monocytes such as psoriasis, rheumatoid arthritis and atherosclerosis[67].

C-reactive protein:

It is a major acute phase reactant. It is synthesised mainly in the liver. Levels of C-reactive protein are increased in conditions of inflammation and they have been established as a risk marker for cardiovascular risk. Its levels have been correlated with insulin resistance [68].

Myeloperoxidase (MPO):

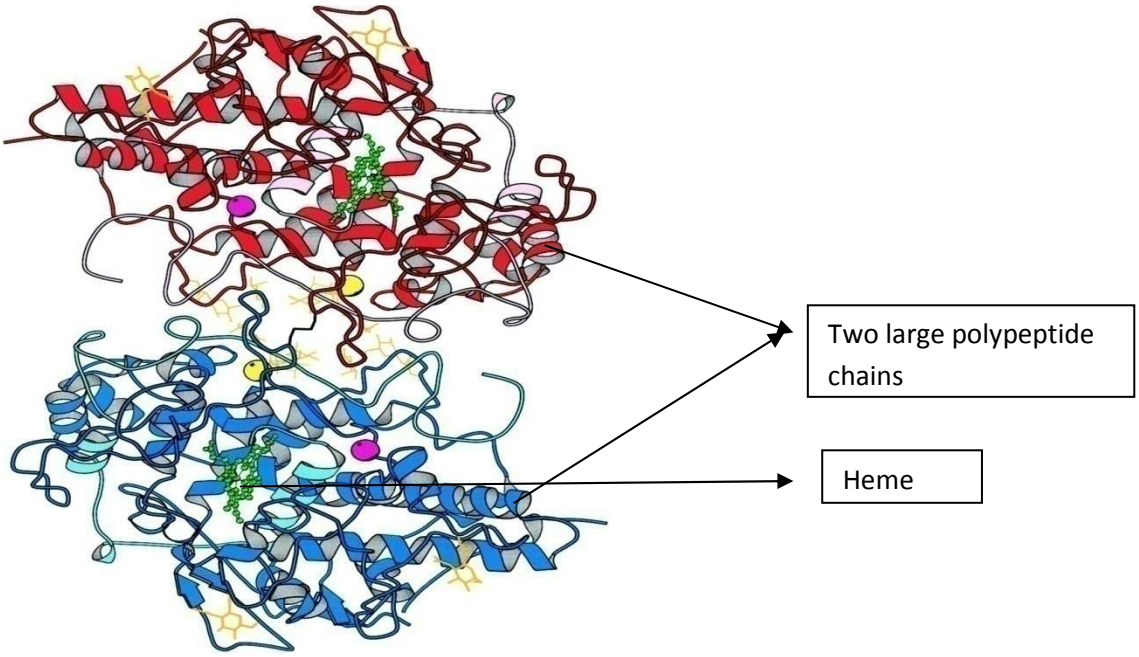
Agner described the purification of an intensely green iron-containing protein from purulent fluid in patients with tuberculous empyema. It was found to exhibit peroxidase activity. Because of its green colour, it was termed initially as verdo-peroxidase. As consequent studies showed that this enzyme expression was restricted to myeloid lineage cells, it was named MPO (EC.1.11.1.7). Chromosome 17 hosts the gene that encodes this enzyme on its long arm 17q22-q23 [69].

Immediately after translation of the MPO gene, a single 80kDa protein is produced. This enters the endoplasmic reticulum and undergoes cleavage of the signal peptide which is made of 41 aminoacids. Further, N-linked glycosylation occurs which leads to incorporation of mannose rich side chains. This leads to the formation of 90kDa apo-pro-MPO.

Apo-pro-MPO is enzymatically inactive because it lacks heme. The enzymatically active pro-MPO is formed by the incorporation of heme.

This enters the Golgi complex. It undergoes a range of post-translational proteolytic modifications to produce the full mature protein. The first proteolytic action cleaves the 125 amino acid pro-peptide to generate a 74 kDa short lived intermediate. Then it undergoes a second cleavage to form two subunits which consists of a 59 kDa heavy subunit and a 13.5 kDa light subunit. These two subunits are linked by covalent bonds with the associated heme group. The native form of MPO has heme in the ferric state. The mature MPO protein is formed by the combination of two heavy and light protomers. They are linked by a disulfide bond between the heavy subunits. The protein after complete post-translational modifications is a homodimer which is of 145 kDa and glycosylated. This homodimer form is the one found in the primary granules of neutrophils and monocytes [70]. The polymorphonuclear neutrophilic cells contain azurophil granules which stores the enzyme MPO.

Figure 3.3: X-Ray crystallographic structure of human MPO



Source: X-ray Crystal Structure and Characterization of Halide-binding Sites of Human Myeloperoxidase at 1.8 Å Resolution. Tristan J. Fiedler, Curt A. Davey, Roger E. Fenna. J. Biol. Chem. 2000, 275:11964-11971.

MPO has been traditionally associated with the process of innate immunity. The complex reactions involved in microbial killing are found associated with MPO. The exceptional ability of this enzyme is to create hypochlorite ion which is formed by the reaction between hydrogen peroxide and chloride ions. The acute inflammatory reaction sets in causing damage to membrane lipids, proteins and nucleic acids. MPO has been concerned in diseases linked with chronic pathological processes, in which oxidative stress and inflammation play dominant roles.

The enzymatic reaction catalysed by MPO is:

$$H_2O_2 + Cl^- \longrightarrow HOCl + H_2O$$

Hypochlorous acid (HOCl) by further reactions interacts with hydrogen peroxide, nitrates to produce highly reactive oxygen and nitrogen species respectively. In addition, HOCl is also found to react with different proteins to form chloramines and modified proteins such as LDL which is modified by the action of HOCl [71]. As these products are highly reactive they induce destruction of the invading pathogens and is almost consistently associated with some degree of harm to the host tissue. These reactions are primarily meant to take place within the limitations of the phagosomes. But occasionally, they are released outside the cell and this brings about destruction of bio-molecules in the adjacent tissue. Even if MPO is fundamental for the defence against invading pathogens, inefficient enzymatic activity could direct to host tissue damage. MPO activity is seen to be increased and being implicated in a wide variety of diseases. The pathological conditions associated with inefficient MPO activity includes the following - cardiovascular disease, malignancies, chronic kidney disease, lung injury and Alzheimer's disease [1].

MPO and endothelial dysfunction:

MPO plays an important role in the commencement, evolution and the complications of atherosclerosis. MPO leads to endothelial dysfunction by making use of the hyperglycemia stimulated H_2O_2 which is derived from vascular non-leukocyte cells by reducing nitric oxide bioavailability [72].

Mechanisms by which MPO promotes atherosclerosis:

A considerable number of epidemiological and clinical studies have demonstrated an association between increased concentrations of MPO and CVD, independent of classical risk factors.

The source of MPO and subsequent formation of reactive species may be triggered by several mechanisms:

1. Inflammation induces recruitment and activation of white blood cells which secretes MPO.
2. Minimally modified LDL particles in the intima triggers the influx of monocytes that mature into resident macrophages, some of which express MPO.
3. Neutrophils in the blood stream are attracted and bound to sites of damaged endothelium. MPO released by these adherent leukocytes is initially bound to the vascular endothelium and subsequently transcytosed to the sub-endothelial matrix.

MPO activity leads to oxidation of LDL cholesterol, which increases its atherogenicity. MPO-induced oxidation of HDL cholesterol can reduce its capacity for reverse cholesterol transport. MPO activity leads to consumption of endothelial derived nitric oxide, which can lead to plaque formation and endothelial dysfunction [73]. Moreover, MPO plays a role in the conversion of a stable to unstable plaque. HOCl produced by MPO induction promotes programmed cell death of the endothelial cell and detachment leading to superficial erosions.

LDL oxidation by MPO:

The oxidative modification of LDL is an early event in atherosclerosis. Oxidized LDL contributes to atherogenesis by promoting cholesterol deposition and transformation of macrophages into foam cells. Retention of LDL in the sub-endothelial space makes LDL a major site for oxidation by pro-oxidants produced by arterial cell wall. Sources of oxidants include NADPH oxidase, xanthine oxidase, lipooxygenases, mitochondrial respiration, uncoupled nitric oxide synthase and MPO. MPO is a protein that is strongly cationic in nature. It can bind to cells in the vascular endothelium, leukocytes and LDL. The association of MPO and LDL enhances the oxidation of this lipoprotein. MPO generates a number of reactive species including HOCl, chloramines, tyrosyl radicals and nitrogen dioxide, which oxidizes the protein, lipid and anti-oxidant

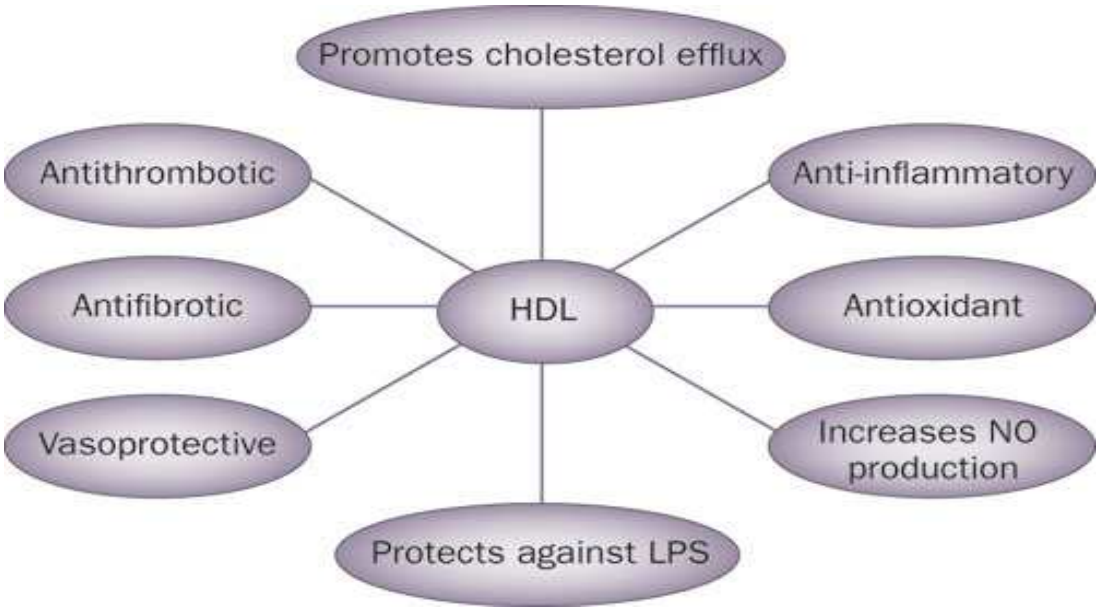
constituents of LDL. Many of the primary oxidation products are unstable and serve as reactive intermediates. This promotes further oxidative modification of LDL and also led to cross-linking and aggregation. The modified tyrosine residues, 3-nitrotyrosine and 3-chlorotyrosine, are among the best characterized of the stable oxidation products of MPO [73]. In contrast to 3- nitrotyrosine, 3-chlorotyrosine is uniquely produced by MPO, and may therefore serve as a unique molecular fingerprint for MPO catalyzed oxidation. The concentration of 3- chlorotyrosine in LDL secluded from atherosclerotic intima was many folds high in circulating LDL [74].

Impairment of HDL functions by MPO:

In addition to playing a central role in cholesterol efflux and reverse-cholesterol transport, HDL also possesses anti-inflammatory and anti-oxidative properties. HDL can prevent or delay oxidation in the vessel wall. In metabolic diseases associated with accelerated atherosclerosis as in DM, HDL particles become functionally defective. Dysfunctional HDL particles lack athero-protective properties and promote pro-inflammatory effects. MPO is implicated in rendering HDL dysfunctional. MPO mediated oxidation of HDL occurs preferentially at the apo A-1 in the HDL molecule. This is evident from the fact that there is a hundred fold increase of both 3-nitrotyrosine and 3- chlorotyrosine in

apo A-1 isolated from circulating HDL in comparison with other circulatory proteins. Selective targeting of apo A-1 is explained by the fact that apo A-1 contains an exact binding site for MPO [75]. Importantly, HDL bound MPO retain its enzymatic activity and binding to HDL protects MPO from cellular uptake and degradation. The HDL which is modified by MPO dependent reactions profoundly increases the affinity of HDL to bind to MPO, which has been projected to lead to a vicious sequence of modifications mediated by MPO at sites of chronic inflammation.

Figure 3.4: Functions of HDL



Abbreviation: NO-Nitric oxide, LPS- Lipopolysaccharide

LDL particles penetrate the intimal layer of the arteries where it may undergo minimal modifications by reactive oxygen species. This consequently leads to the induction of monocytes to migrate into the

vessel wall. They undergo differentiation into macrophages. In the macrophages, scavenger receptors recognise the modified oxidized LDL and leads to its uptake. The excess uptake of the modified LDL leads to formation of foam cell. During conditions of inflammation, MPO gets released by macrophages and catalyzes the production of reactive species using different substrates such as chloride, thiocyanate or nitric oxide with H_2O_2 as co-substrate. This results in depletion of nitric oxide which leads to an impairment in vasodilatation. The reactive species formed can also oxidize LDL particles to form oxidized LDL, renders HDL to lose its functions by enhancing the formation of oxidized HDL, which impairs the protective effect of HDL on LDL-particles and in addition inhibiting reverse-cholesterol transport.

MPO reduces bioavailability of nitric oxide:

In the endothelium, synthesis of nitric oxide by the endothelial isoform of nitric oxide synthase helps in the regulation of blood vessel tone. The nitric oxide produced acts as a powerful vasodilator. In addition it is found to suppress the endothelial binding of cells in the circulation. It also inhibits smooth muscle cell proliferation in the vessel wall. These actions point towards the fact that nitric oxide is an essential element for the maintenance of vascular homeostasis. There is an increase in the rate of atherosclerosis and impairment of vascular functions due to inadequate

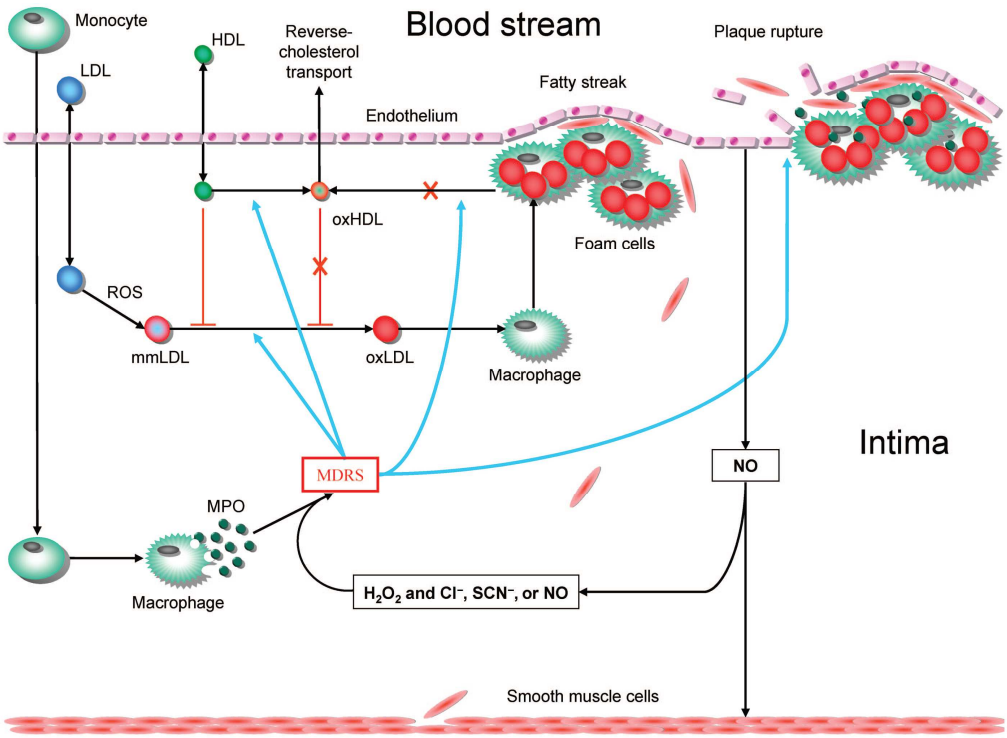
production or increased removal of nitric oxide. MPO is found to reduce the bioavailability of nitric oxide by a number of mechanisms. One proposed mechanism is that peroxidases can also use nitric oxide as a substrate and thus one of the members of the peroxidase family namely MPO serves as a catalytic sink for nitric oxide [76]. Second, reactive substances produced by MPO leads to the removal of nitric oxide which further reduce its bioavailability. One other mechanism suggests that hypochlorous acid reacts with arginine, one of the nitric oxide synthase substrate and forms chlorinated arginine compounds. These compounds are inhibitors of all isoforms of the nitric oxide synthase enzyme thus lead to impaired endothelium-dependent relaxation [77]. Finally, it has been established that nitric oxide synthase uncoupling is induced by hypochlorous acid. This turns it into a superoxide-producing enzyme. Even though the relative impact of these mechanisms is currently indefinite, it is clear that MPO is found to deplete nitric oxide in the endothelial vessel walls by both catalytic as well as non-catalytic mechanisms.

MPO and plaque vulnerability:

Acute cardiovascular events are found to be induced by the crucial processes such as destabilization of the atherosclerotic plaque and rupture. MPO activates the metalloproteinases which thereby leads to

weakening of the fibrous cap of the plaque, thus leading to its destabilization. Plaque injury activates neutrophils, which leads to MPO release. Malle *et al* [78] observed co-localization of MPO and hypochlorite customized proteins in lesions of human atherosclerotic plaque. Sugiyama *et al* [79] reported that there was little or no MPO in macrophages isolated from fatty streaks of the atherosclerotic plaque whereas macrophages in plaques that undergo erosions and rupture are rich in MPO.

Figure 3.5: Adverse effects of MPO in the vasculature



Importance of MPO in DM:

Inflammation has been recognized as the underlying basis of chronic diseases including Type-2 DM. It has a fundamental role in mediating all the phases of atherosclerosis, from the commencement stage through progression and ultimately leading to thrombotic complications.

In Type-2 diabetes, inflammation and activation of monocytes play a predominant role in reducing insulin sensitivity and also contributes to the loss of insulin secretion by islet cells. Hyperglycemia associated with diabetes can lead to alterations in the macromolecules which forms AGE and others, which can enhance the formation of pro-inflammatory markers like cytokines in vascular endothelium.

Free radicals are formed excessively in diabetes. This excess production has been attributed to oxidation of glucose, glycation of proteins and oxidative breakdown of glycated proteins. The raised levels of reactive oxygen species and associated reduction of antioxidant defence mechanisms leads to cellular damage. It also leads to an increase in the propensity for lipid peroxidation and development of insulin resistance. Inflammation and oxidative stress can accelerate the process of atherosclerosis in DM. MPO, a pro-oxidant enzyme, released from the

granules of leukocytes, monocytes and macrophages from the inflammatory sites can stimulate increased production of reactive oxygen species which can cause oxidative damage to the endothelium and vessel wall. Cardiovascular disease accounts for about 70% of all deaths in patients with diabetes. This increase has been ascribed to accelerated atherosclerosis and endothelial dysfunction which are due to the inflammatory and oxidative stress response in diabetic patients.

Evidence regarding MPO activity in diabetes is limited and inconsistent, demonstrating both higher and lower levels of MPO in different tissues and clinical situations [80, 81, 82]. However, it is known that endothelial dysfunction develops early in DM, preceding clinically detectable atherosclerosis, and that diminished nitric oxide levels and enhanced oxidative stress are important determinants in the pathogenesis of diabetic vascular complications. Type-2 DM is associated with mildly increased levels of MPO, independent of other clinical considerations.

This relationship may add-up to the accelerated progression of atherosclerosis in diabetes. MPO plays an important role in the initiation, progression and the rupture of plaque seen in atherosclerosis. Previous studies have specifically focused on the relation between MPO levels and the presence of endothelial dysfunction in diabetes.

These studies showed a significantly higher MPO levels in diabetes. But some studies also showed no correlation of MPO level in diabetes [83]. Not many works are there to see the relationship between MPO and HbA_1c and also with lipid abnormalities. So this study is undertaken to see the plasma MPO activity, lipid profile and HbA_1c and also to see the relationship between them.

MATERIALS AND METHODS

The study was conducted for a period of one year at PSG Institute of Medical Sciences and Research, Coimbatore. An informed consent was taken from the patients before sample collection. Ethical clearance was obtained from the Institutional Human Ethics Committee.

It is a cross-sectional study including two groups. Non-diabetic healthy individuals served as controls (n=50). The cases included patients diagnosed with type-2 DM satisfying the inclusion and exclusion criteria. (n=50). Cases were selected from diabetic patients attending Endocrinology and Medicine OPD. Non-diabetic controls were included from those attending the master health check-up. The patients recruited in the study were randomly selected.

Inclusion criteria:

1) Age>30 years

2) Patients diagnosed as type 2 DM as per WHO criteria not less than one year duration

Exclusion Criteria:

1) Chronic kidney disease.

2) Coronary artery disease.

3) Previous vascular disease.

4) Auto-immune disease.

5) Hypertension.

6) Patients on treatment with statins.

Collection of blood samples:

5 ml of venous blood sample was collected after a period of 12 hours overnight fasting HbA_1c, lipid profile and plasma MPO activity were estimated in the sample.

Anthropometric measurements like weight and height were also recorded.

PLASMA MPO ACTIVITY:

Method:

Serum MPO was estimated by spectrophotometric method using O-dianisidine dihydrochloride as a substrate [84].

Principle:

This method is based on the oxidation of O-dianisidine (colourless chromogen) by hydrogen peroxide to form a yellowish orange coloured oxidized product.

MPO

O-dianisidine + H_2O_2 ⟶ Oxidized O-dianisidine (Yellowish orange Coloured product) + H_2O

The increase in absorbance at 470nm due to the formation of oxidised o-dianisidine was measured.

Reagents:

All the chemicals were of analytical grade.

1. Stock phosphate buffer: Consist of solution A and solution B

Solution A – 1M Dipotassium hydrogen phosphate solution. Prepared by mixing 17.41g K_2HPO_4 in 100 mL distilled water.

Solution B – 1M Potassium dihydrogen phosphate solution. Prepared by mixing 13.6g KH_2PO_4 in 100 mL of distilled water.

2. Working phosphate buffer: Prepared by mixing 13.2mL of solution A with 86.8mL of solution B and made up to 500mL with distilled water. The pH of the working buffer was maintained at 6.

3. Substrate: O-Dianisidine Dihydrochloride (5mg/L). Substrate must be freshly prepared by mixing 5mg of O-Dianisidine dihydrochloride (Sigma-Aldrich product) in 1mL of distilled water.

4. HydrogenPeroxide: 30% Hydrogen peroxide solution was used directly.

Procedure:

- 2.8mL of working buffer was taken in a test tube.
- To this 0.1ml O-Dianisidine dihydrochloride (5mg/mL) was added.
- To this solution 25 μL of sample was added.
- The solution was taken in a cuvette.
- Spectrophotometer was set at 470nm and calibrated with distilled water as blank.
- 100 μL of Hydrogen peroxide was added to the reacting mixture in the cuvette and kept in spectrophotometer.
- Optical density was measured at 30 second intervals for 2 minutes.

Calculation:

One unit (U) of MPO activity was defined as that degrading 1 μmol of hydrogen peroxide per minute at 25^0C.

$$\text{Activity of MPO in the given sample} = \frac{\Delta OD/\text{minute} \times 3.025}{0.025 \times 11300 \times 10^6}$$

$$= \Delta OD/\text{minute} \times 10676 \text{ U/L}$$

Where ΔOD = (Final optical density – initial optical density)/2

3.025 is reaction volume in mL

0.025 is the sample volume in mL

11300 is the molar absorptivity in Mol/cm

PLASMA GLUCOSE

Method:

Enzymatic reference method with hexokinase.

Principle:

The phosphorylation of glucose by ATP to form glucose-6 phosphate and ADP is catalysed by Hexokinase (HK). This is followed by the action of another enzyme, glucose-6 phosphate dehydrogenase (G6PDH). This enzyme catalyzes oxidation of glucose-6 phosphate by NAD^+ to form NADH.

Reaction:

$$\text{D-glucose + ATP} \xrightarrow{\text{HK}} \text{D-glucose-6 phosphate + ADP}$$

$$\text{D-glucose-6 phosphate + } NAD^+ \xrightarrow{\text{G6PDH}} \text{D-6 phosphogluconate } + NADH + H^+$$

The concentration of NADH formed is directly proportional to the glucose concentration. It is determined by measuring the increase in absorbance at 340nm.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	340/409 nm
Test range	0-40 mmol/L (0-720 mg/dL)
Unit	mmol/L

SERUM TOTAL CHOLESTEROL

Method:

Enzymatic colorimetric test.

The method involves use of enzymes cholesterol esterase, cholesterol oxidase and peroxidase.

Principle:

Cholesterol esters are cleaved by the action of cholesterol esterase (CE) to yield free cholesterol and fatty acids. Cholesterol oxidase (CHOD) in a second reaction catalyzes the oxidation of cholesterol to

cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase (POD), the hydrogen peroxide formed effects the oxidative coupling of phenol and 4-aminoantipyrine to form a red quinone-imine dye.

Reaction:

$$\text{Cholesterol esters} + H_2O \xrightarrow{\text{CE}} \text{Cholesterol} + \text{R-COOH}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHOD}} \text{Cholest-4-ens-3-one} + H_2O_2$$

$$2\ H_2O_2 + 4\ \text{AAP} + \text{Phenol} \xrightarrow{\text{POD}} \text{Quinone-imine dye} + H_2O$$

The color intensity of the dye formed is directly proportional to the cholesterol concentration. It is determined by measuring the increase in absorbance at 512 nm.

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	512/659 nm
Test range	0-20.7 mmol/L (0-800 mg/dL)
Unit	mmol/L

SERUM TRIGLYCERIDES:

Method:

Enzymatic, colorimetric method.

Involves enzymes glycerol phosphate oxidase and 4-aminophenazone.

Principle:

Lipoprotein lipase (LPL) hydrolyzes the triglycerides to glycerol and fatty acids. Glycerol is phosphorylated to glycerol-3-phosphate by ATP catalyzed by glycerol kinase (GK). The oxidation of glycerol-3-phosphate is catalyzed by glycerol phosphate oxidase (GPO) to form dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase (POD), hydrogen peroxide effects the oxidative coupling of 4-chlorophenol and 4-aminophenazone to form a red-coloured quinoneimine dye, which is measured at 512 nm. The increase in absorbance is directly proportional to the concentration of triglycerides in the sample.

Reaction:

$$\text{Triglycerides} \xrightarrow{\text{LPL}} \text{Glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3 phosphate + ADP}$$

GPO

Glycerol -3 phosphate + O_2 ⟶ Dihydroxy acetone Phosphate+H_2O_2

POD

2 H_2O_2 + 4 aminophenazone ⟶ Quinoneimine dye +4H_2O+4Chlorophenol

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R-S
Direction of Reaction	Increase
Wavelength A/B	512/659 nm
Test range	0-10 mmol/L (0-885 mg/dL)
Unit	mmol/L

SERUM HDL CHOLESTEROL:

Method:

Homogenous enzymatic colorimetric assay.

Principle:

In the presence of magnesium ions, dextran sulphate selectively forms water-soluble complexes with LDL, VLDL and chylomicrons which are resistant to PEG modified enzymes. The cholesterol

concentration is initially determined by enzymatic methods using cholesterol esterase and cholesterol oxidase coupled with PEG to the amino groups. Cholesterol esterase breaks down the cholesterol esters into free cholesterol and fatty acids. The enzyme cholesterol oxidase oxidizes the cholesterol in the presence of oxygen to Δ4-cholestenone and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide generated reacts with 4-aminoantipyrine and HSDA to form a purple-blue dye. The intensity of colorformed by the blue quinoneimine dye formed is directly proportional to the cholesterol concentration which is measured photometrically.

Reaction:

$$\text{HDL Cholesterol ester} + H_2O \xrightarrow{\text{PEG cholesterol esterase}} \text{HDL Cholesterol} + \text{RCOOH}$$

$$\text{HDL-Cholesterol} + O_2 \xrightarrow{\text{PEG Cholesterol oxidase}} \Delta\text{ 4-cholestenone} + H_2O_2$$

$$2H_2O_2 + \text{4 aminoantipyrine} + \text{HSDA} + H^+ \xrightarrow{\text{POD}} \text{Purple blue Pigment} + 4H_2O$$

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	583/659 nm
Test range	0-3.12 mmol/L (0-120 mg/dL)
Unit	mmol/L

SERUM LDL CHOLESTEROL:

Method:

Homogeneous enzymatic colorimetric assay.

Principle:

This method is based on the property of selective micellary solubilization of LDL-cholesterol by a non-ionic detergent. It also utilises the interaction of a sugar compound with lipoproteins such as VLDL and chylomicrons. Addition of a detergent to the enzymes used for cholesterol estimation (cholesterol esterase - cholesterol oxidase coupling reaction), increases the relative reactivities of cholesterol in the lipoprotein fractions in the following order: HDL < chylomicrons < VLDL < LDL. The activity of the enzymes used in the cholesterol estimation is reduced in

the presence of magnesium ions and a sugar. The selective estimation of LDL-cholesterol in serum is enabled by the reaction of a sugar molecule with a detergent. In the presence of oxygen, cholesterol is oxidized by cholesterol oxidase to Δ4-cholestenone and hydrogen peroxide. The intensity of the blue quinoneimine dye formed is directly proportional to the concentration of LDL cholesterol. It is determined by monitoring the increase in absorbance at 583 nm.

Reaction:

$$\text{LDL-Cholesterol ester} + H_2O \xrightarrow[\text{(Selective micellary solubilisation)}]{\text{Detergent cholesterol esterase}} \text{Cholesterol} + \text{Free fatty acid}$$

$$\text{LDL-Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \Delta 4\text{-cholestenone} + H_2O_2$$

$$2H_2O_2 + 4\ \text{aminoantipyrine} + \text{HSDA} + H^+ \longrightarrow \text{Purple blue pigment} + 4H_2O$$

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	R1-S-SR
Direction of Reaction	Increase

Wavelength A/B	583/659 nm
Test range	0-14.2 mmol/L (0-550 mg/dL)
Unit	mmol/L

Glycated haemoglobin: (HbA_1c)

Method:

Turbidimetric inhibition immunoassay

Principle:

EDTA tube collected whole blood specimen is hemolysed after collection. Total hemoglobin and HbA_1c concentrations are determined. Total hemoglobin is measured colorimetrically. The percentage of HbA_1c is determined immunoturbidimetrically. The ratio of both these levels gives the final percent HbA_1c result.

EDTA collected blood is hemolysed with hemolysis reagent in the predilution cuvette. This leads to reduction in osmotic pressure which lyses the erythrocytes. Lysis of erythrocytes releases the haemoglobin. It is degraded by the proteolytic action of the enzyme pepsin. This renders the beta-N terminal structures more available for the immunoassay.

In the hemolysate, a colorimetric method helps in the determination of total haemoglobin. This is done on the basis of production of a

brownish-green chromophore. This occurs in alkaline detergent solution using a cyanide free method.

The intensity of colour is directly proportional to the total hemoglobin concentration in the sample. The concentration is determined by sensing the increase in absorbance at 552nm. A fixed factor that is obtained from the primary calibrator chlorohemin calculates the test results.

Turbidimetric method to measure HbA_1c is done using monoclonal antibodies which are present attached to latex particles. The monoclonal antibodies bind the amino terminal fragments of HbA_1c. The unbound free antibodies agglutinate with an artificial polymer. The change in turbidity is inversely related to the quantity of bound glycated proteins. This is measured turbidimetrically at 552nm.

The final result is expressed as percentage HbA_1c. It is calculated from the ratio of HbA_1c/Hb as given below:

According to IFCC

$HbA_1c\ (\%) = (\ HbA_1c\ /\ Hb) \times 100$

According to DCCT/NGSP

$HbA_1c(\%) = (HbA_1c/Hb) \times 87.6 + 2.27$

Test definition:

Mode of measurement	Absorbance
Mode of calculation of absorbance	Endpoint
Mode of reaction	D-R1-S-SR
Direction of Reaction	Increase
Wavelength A/B	552nm
Test range	1.4-4.7µmol/L (2.4-75.8mg/dL)
Unit	µmol/L

STATISTICAL ANALYSIS

Baseline characteristics are measured as Mean ± SD. Significance is assessed at 5% level of significance. Student‘t’ test (two tailed, independent) and Chi-square test has been used to find the significance of study parameters. ROC curve analysis is performed to find the sensitivity and specificity of the diagnostic test. Pearson correlation was done to establish correlation between MPO and other relevant parameters such as lipid profile and HbA_1c.

RESULTS

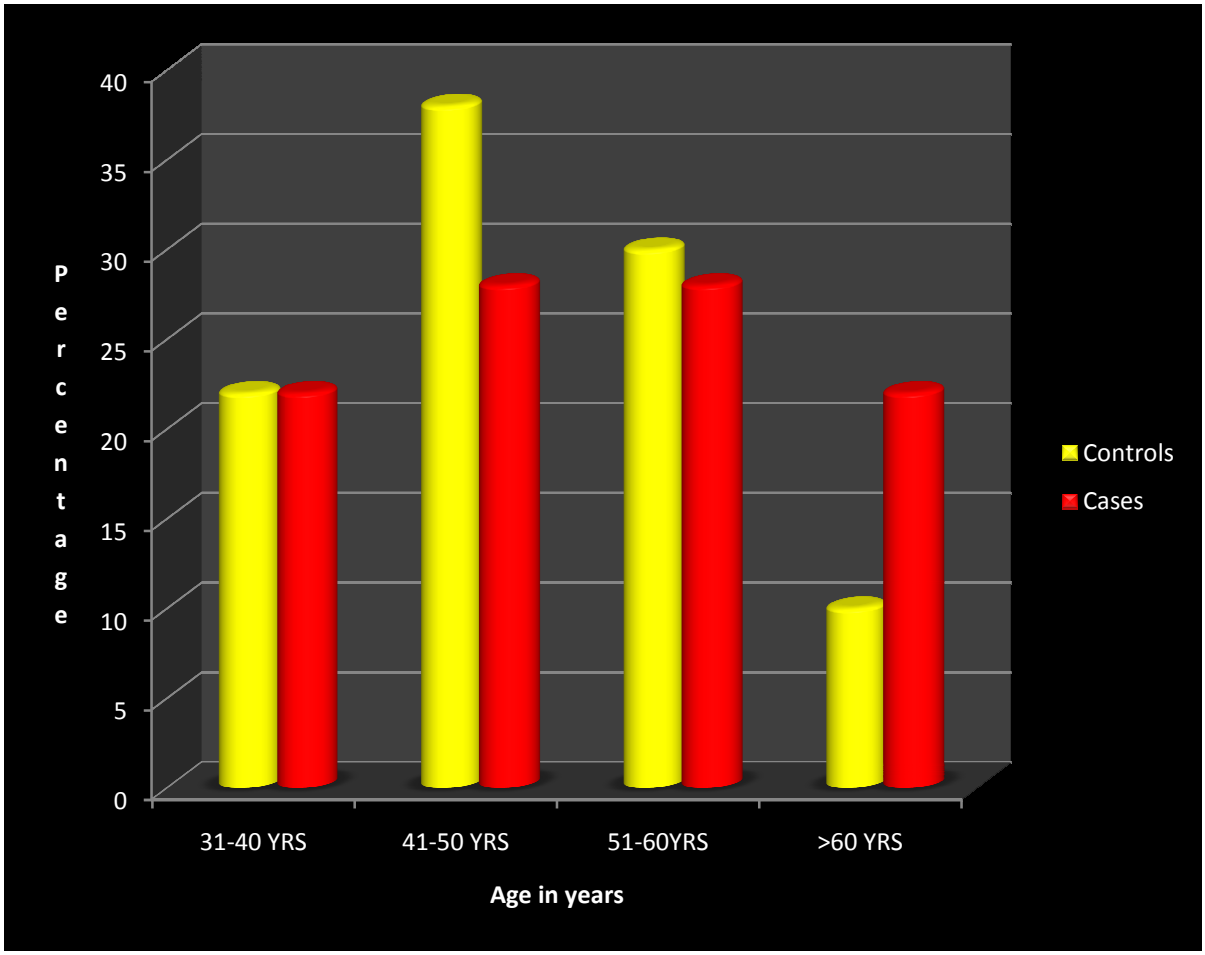
This study was done to estimate the plasma MPO levels in patients with type-2 DM and to compare the same with healthy non-diabetic control patients. In addition, plasma MPO activity was correlated with lipid profile and HbA_1c.

AGE DISTRIBUTION OF THE STUDY POPULATION:

Table 6.1: Distribution of cases and controls as per age group.

Age	Controls		Cases	
	Number	Percent	Number	Percent
31-40 yrs	11	22.0	11	22
41-50 yrs	19	38.0	14	28
51-60 yrs	15	30.0	14	28
>60 yrs	5	10.0	11	22

Figure 6.1: Age distribution of the study population



The age distribution of control subjects and diabetic cases are shown in the Figure 6.1. There is no significant difference between the mean age of diabetic cases and controls.

Table 6.2: Mean Age of Study Population

Age in years		
	Controls	Cases
Mean ±SD	48.12±8.56	51.30±11.41
p value	0.16 (Not significant)	

Table 6.3: Gender Distribution of Study Population:

	Controls	Cases
Number of males	29	33
Number of females	21	17
Total	50	50

Figure 6.2: Gender distribution in control group

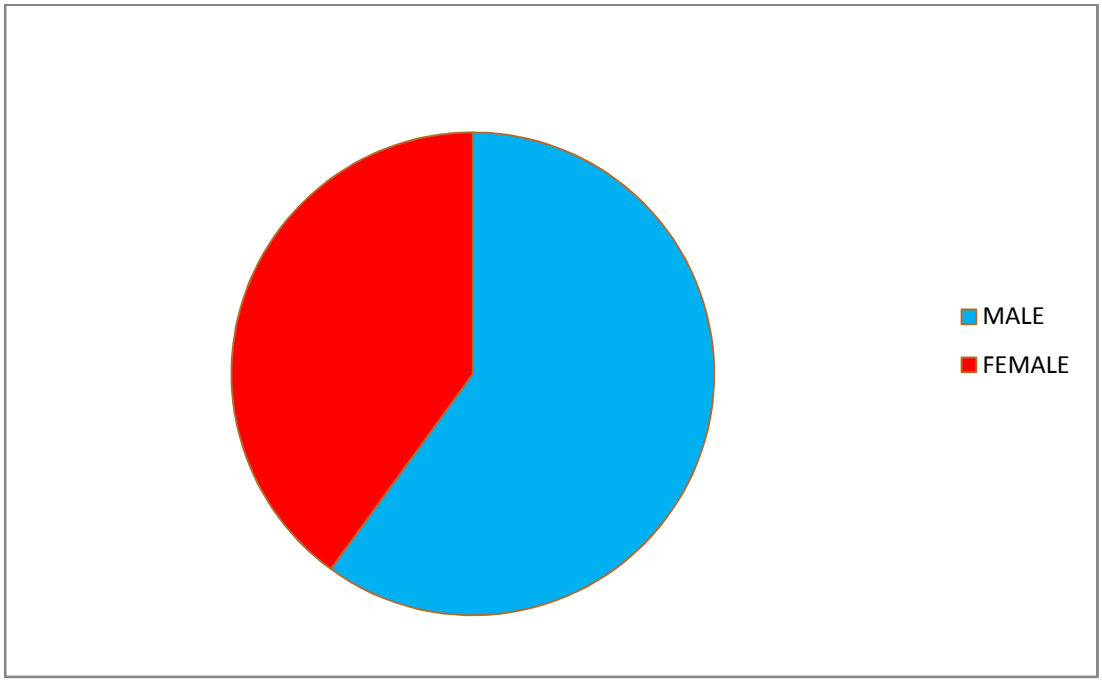


Figure 6.3: Gender distribution in diabetic patients group

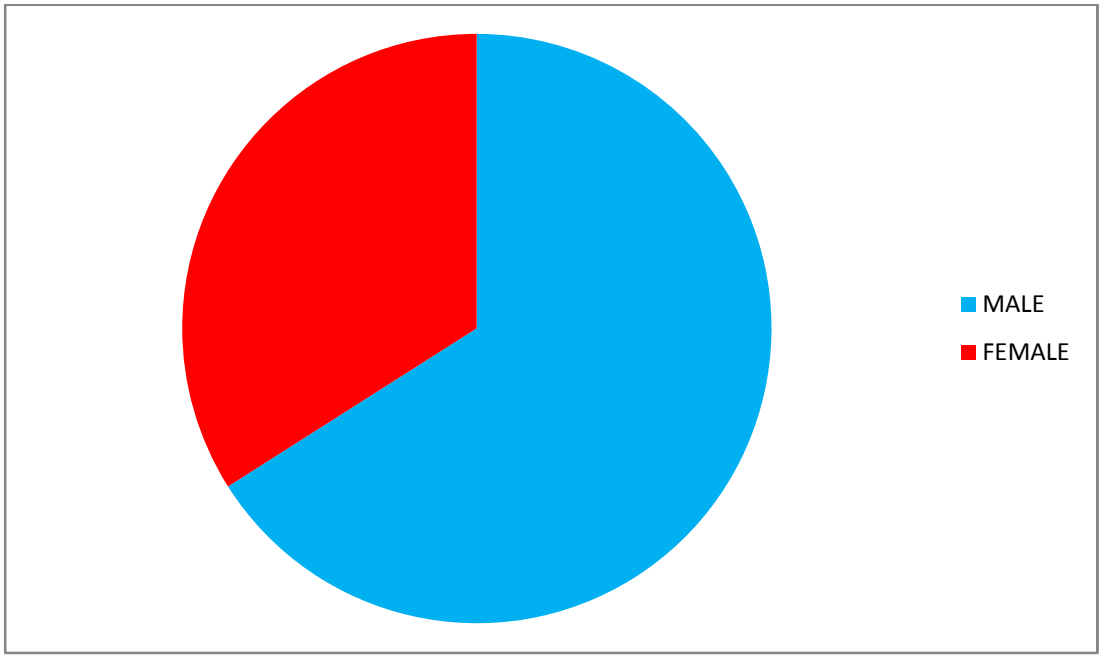


Figure 6.4: Distribution of BMI in the study population

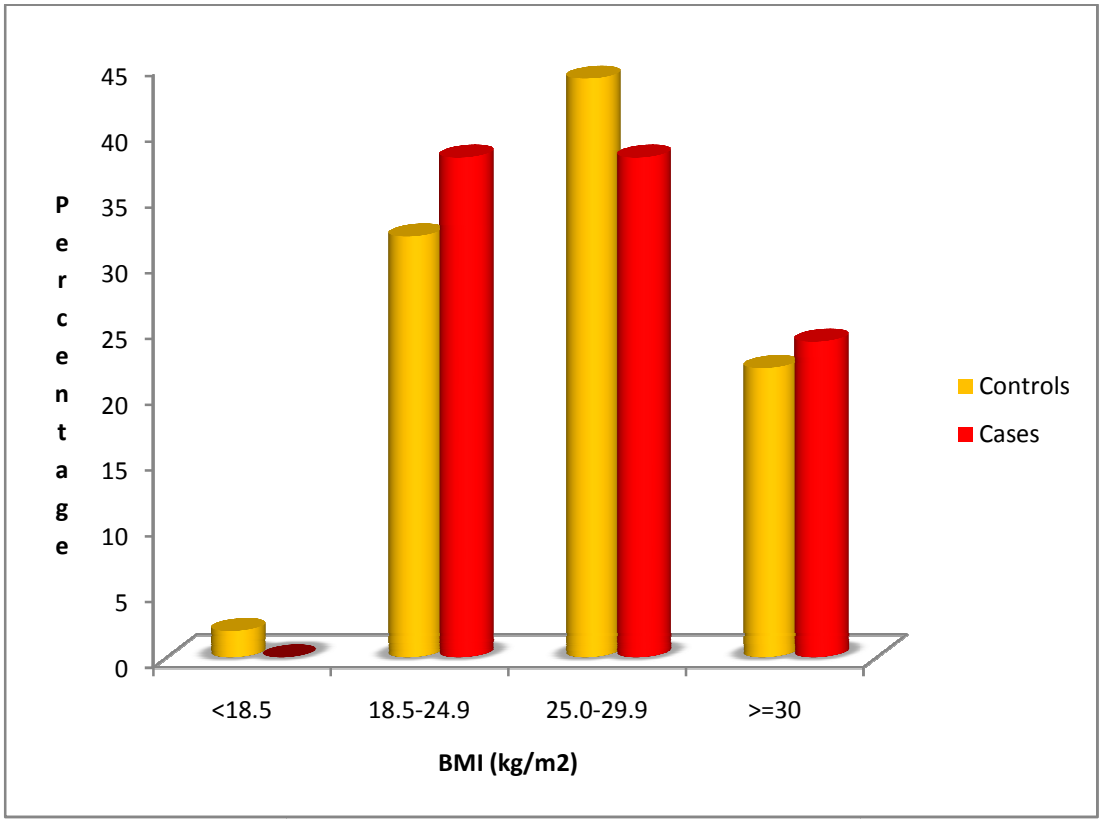


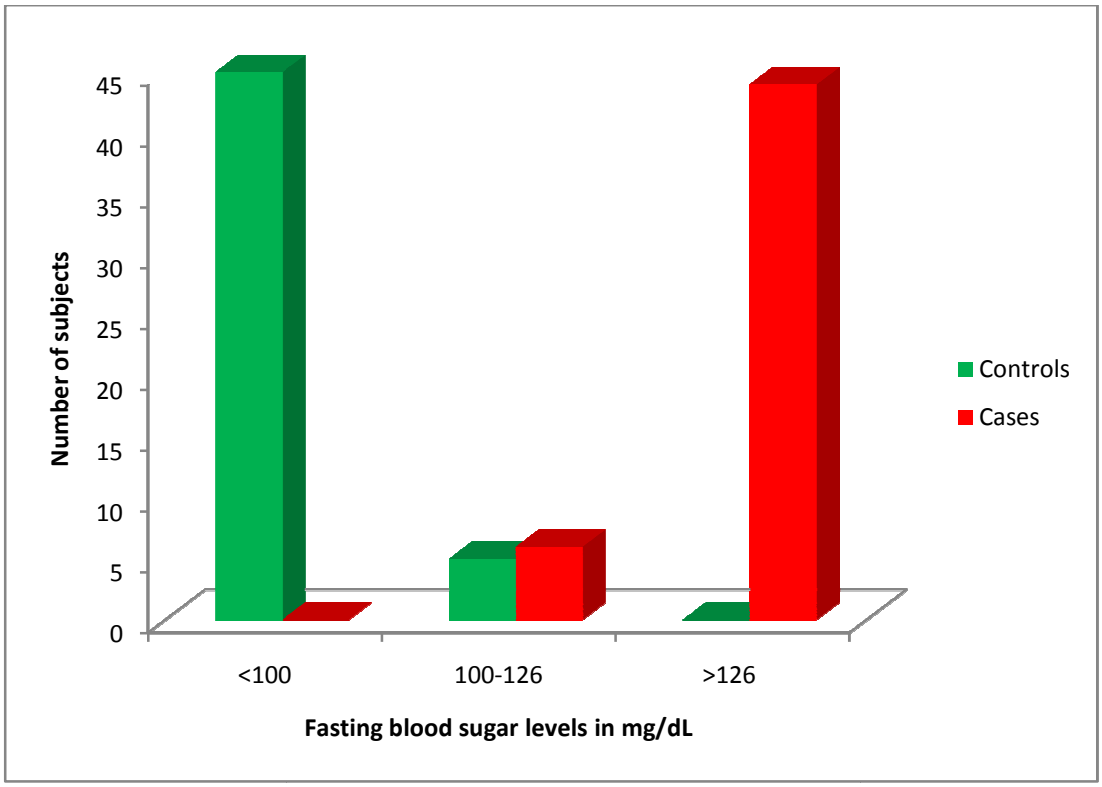
Table 6.4: Mean BMI of study groups

Body mass index (kg/m^2)		
	Controls	Cases
Mean ± SD	26.41±4.40	27.06±4.79
p value	0.48(Not significant)	

The mean BMI (kg/m^2) in non-diabetic controls is about 26.41 ± 4.40 whereas in diabetic cases it is around 27.06 ±4.79. The difference in

BMI between the study groups is not statistically significant (p value of 0.48).

Figure 6.5: Fasting blood sugar distribution in the study groups



The distribution of the fasting blood sugar concentrations in both the study groups is presented graphically in Figure 6.5. The mean blood glucose levels in the control group are 93.14±8.15 mg/dL whereas it is 204.14±75.64mg/dL in the diabetic cases group. All the diabetic cases in the study have fasting blood sugar (FBS) greater than 126 mg/dL.

Table 6.5: Comparison of lipid profile between the study groups

	Controls	Cases	p value
	Mean ± SD		
Serum cholesterol (mg/dL)	182.2± 36.86	189.70±35.24	0.3
Serum triglycerides (mg/dL)	128.38±53.19	164.16±90.55	0.02*
Serum HDL (mg/dL)	45.02±13.17	43.00±9.73	0.39
Serum LDL (mg/dL)	124.18±13.17	120.4±31.00	0.5

*p <0.05 – Statistically significant

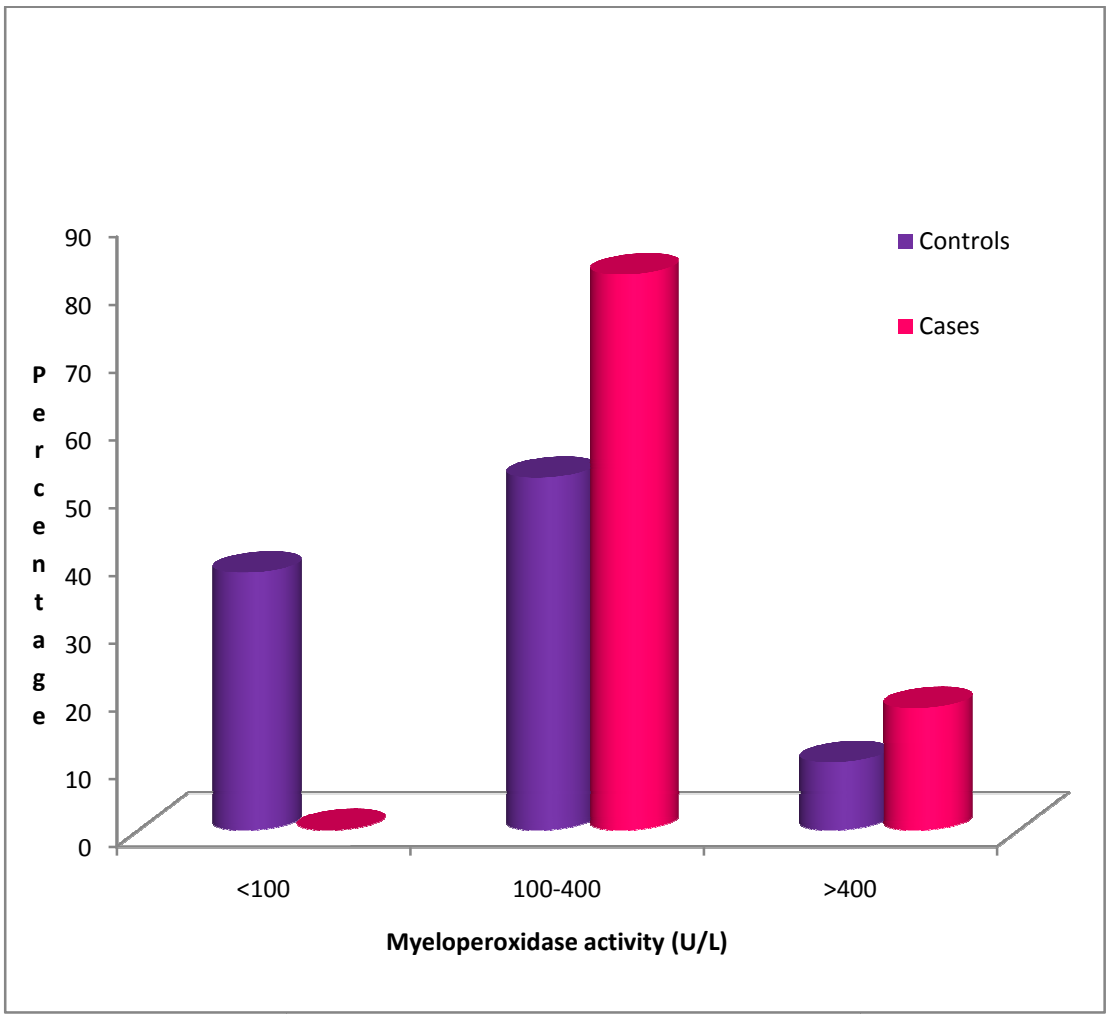
The mean of the lipid profile of cases and controls are compared in Table 6.5. There was statistically significant difference in the triglyceride levels of the diabetic cases when compared with the non-diabetic controls. The other parameters of the lipid profile did not differ significantly between the two study groups.

Table 6.6: Plasma MPO activity in cases and controls.

PLASMA MPO ACTIVITY (U/L)		
	Controls	Cases
Mean ± SD	188.41±142.73	317.17±247.73
p value	0.0019*	

*p <0.05 – Statistically significant

Figure 6.6: MPO activity in the study population



Plasma MPO activity in diabetics is significantly higher than in the controls with p value of 0.0019 ($p < 0.05$ – statistically significant). The mean MPO activity in diabetic patients is 317.17±247.73 U/L. It is found that none of the diabetic patients had MPO activity of less than 100 U/L.

Figure 6.7: MPO activity vs BMI in controls

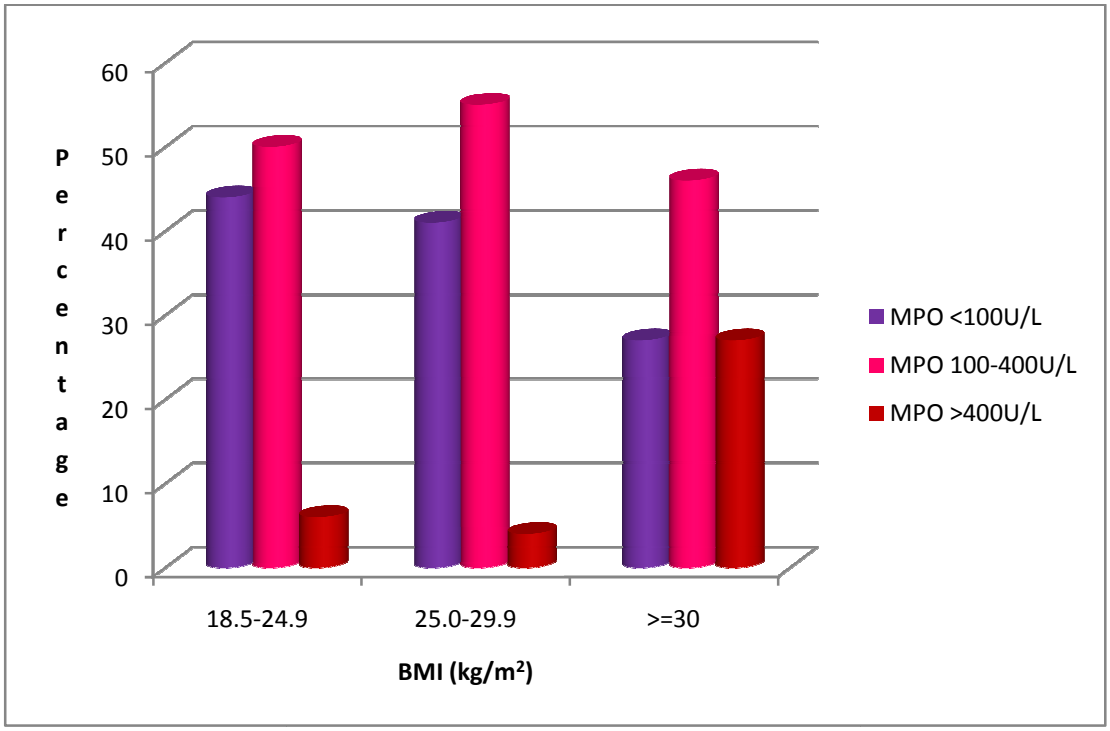
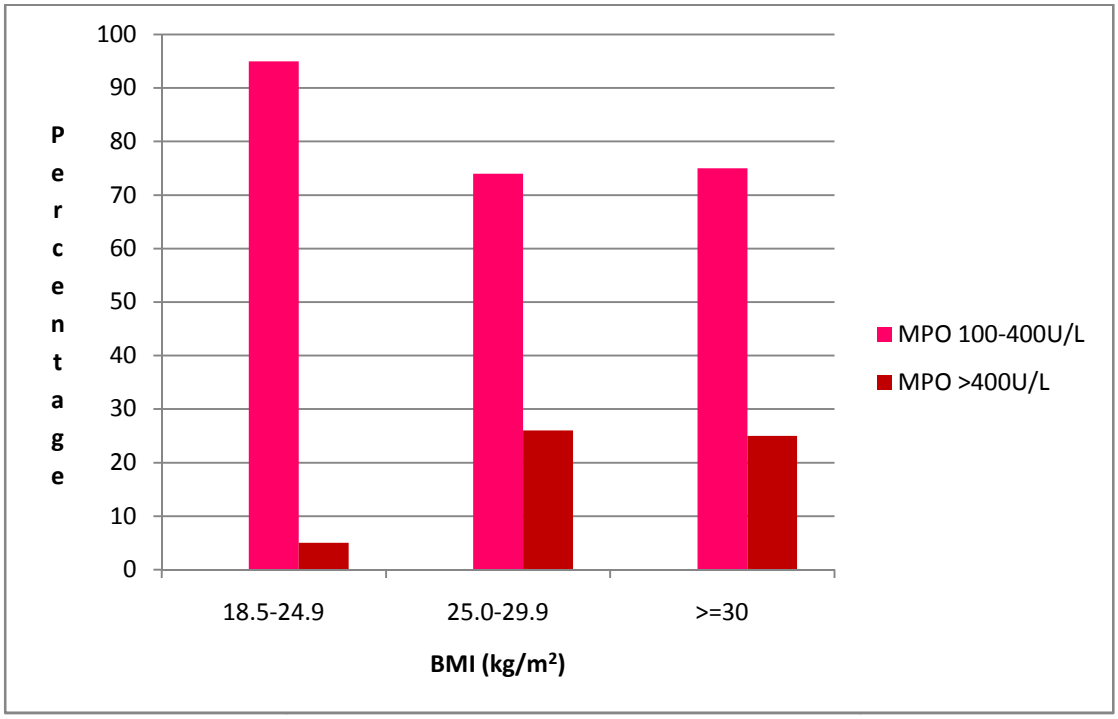


Figure 6.8: MPO activity vs BMI in cases



In the control group, MPO activity is found to increase with increase in Body mass index. MPO activity of >400U/L is highest in the individuals with BMI≥30kg/m^2 as shown in Figure 6.7

Increased MPO activity of >400U/L is found to increase with an increase in BMI.

Plasma MPO activity with respect to gender is analysed and presented in the following figures.

Figure 6.9: MPO activity vs gender in controls

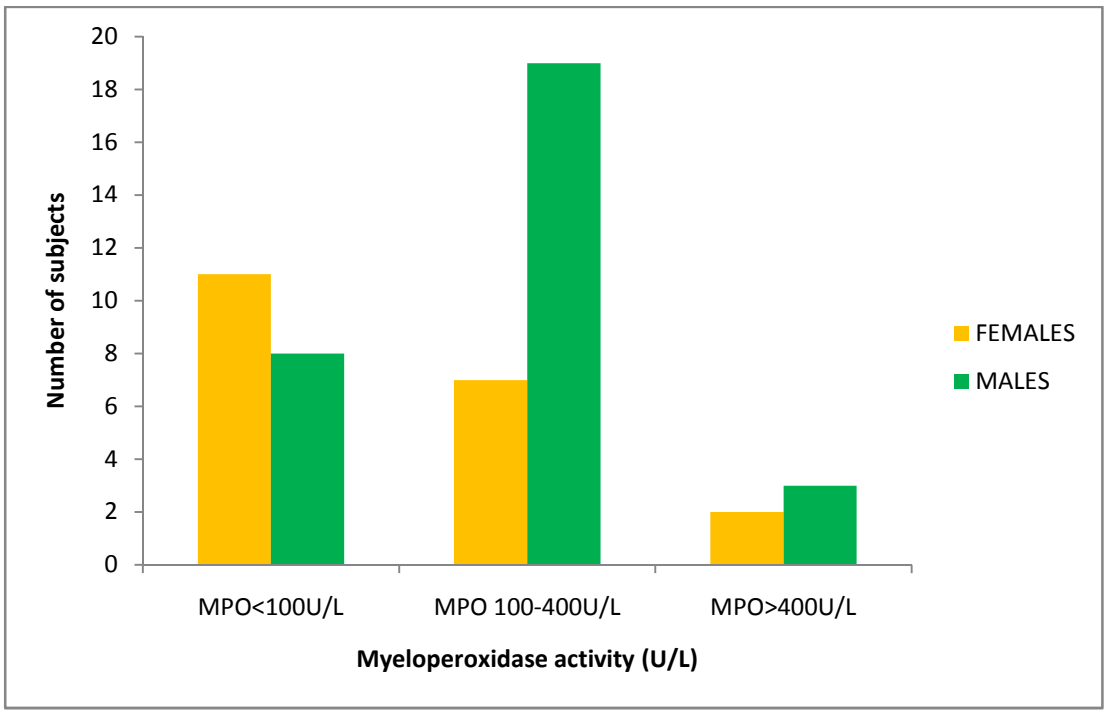
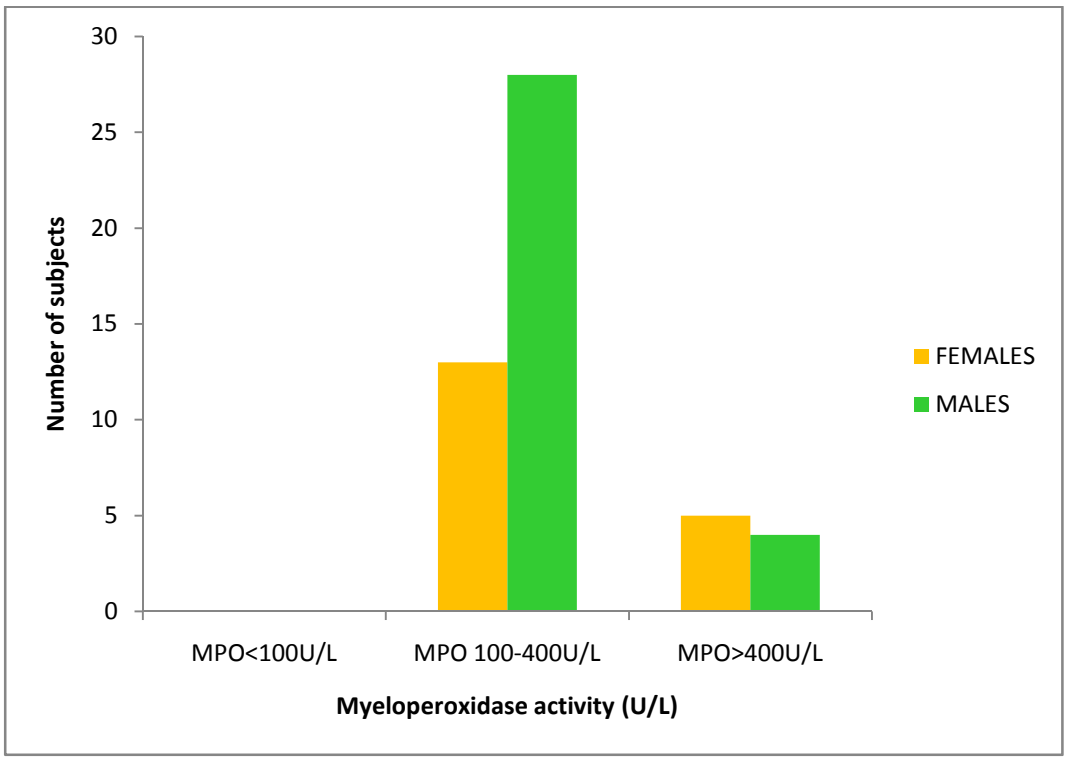


Figure 6.10: MPO activity vs gender in cases



Though MPO activity is found to be higher in males when compared to females in the two groups as shown in Figure 6.9 and Figure 6.10, there is no significant statistical difference in MPO activity in both the genders in the study population (p value of 0.19 in control group and 0.38 in cases group)

Correlational analysis between MPO activity and BMI (expressed as kilograms/metresquare), serum cholesterol (expressed as mg/dL), serum triglyceride (expressed as mg/dL), serum HDL (expressed as mg/dL), HbA_1c (measured as %) in the diabetic patients is done using Pearson's correlational testing and the results are presented in Table 6.7.

Table 6.7: Pearson's correlation analysis between MPO activity and BMI, Serum Cholesterol, Serum Triglycerides, Serum HDL, HbA_1c in cases

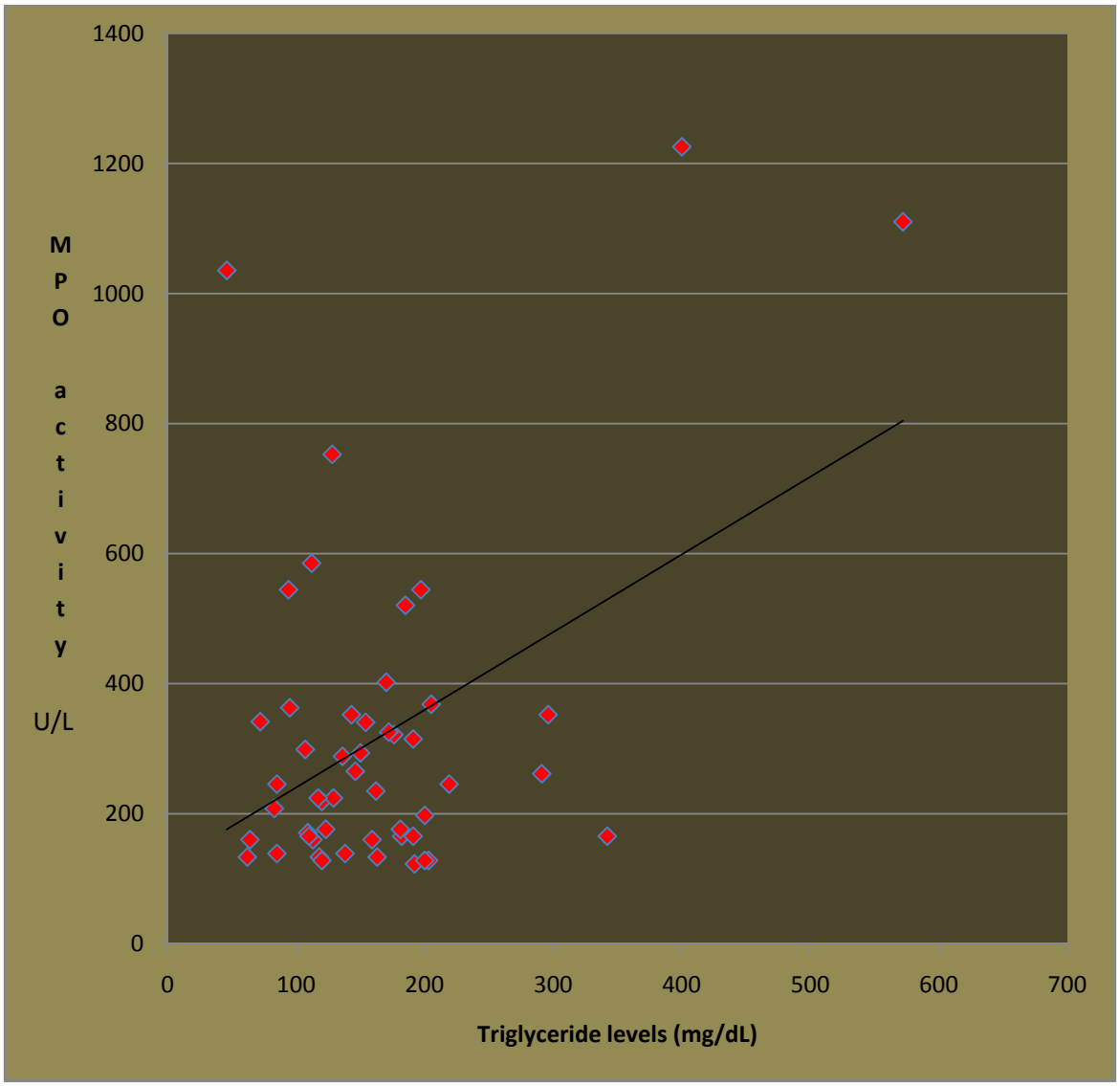
	r value	p value
MPO activity & BMI	0.154	0.286
MPO activity & Serum Cholesterol	0.272	0.056
MPO activity & Serum HDL	-0.091	0.345
MPO activity & Serum Triglycerides	0.437	0.002*
MPO activity & HbA1c	0.235	0.1

*$p < 0.05$ – Statistically significant

The Pearson correlation of MPO activity with total cholesterol, triglyceride, BMI and HbA_1c is shown in Table 6.7. In the present study, a significant positive correlation is observed between MPO activity and serum triglyceride levels. The correlation of MPO activity with BMI,

Cholesterol and HbA_1c is not very relevant. The moderate correlation between plasma MPO activity and serum triglyceride levels are represented in the scatter diagram Figure 6.11.

Figure 6.11: Scatter plot showing the correlation between triglyceride levels and MPO activity



Further ROC curve has been used to find sensitivity and specificity of serum MPO activity in diabetic patients. This curve is compared with ROC curves for serum triglyceride levels, serum HDL levels and serum LDL levels. (Figure 6.12) The area under the curve for each of these parameters is presented in Table 6.8.

Figure 6.12: Comparison of ROC curves of MPO activity, serum triglycerides, serum HDL, serum LDL

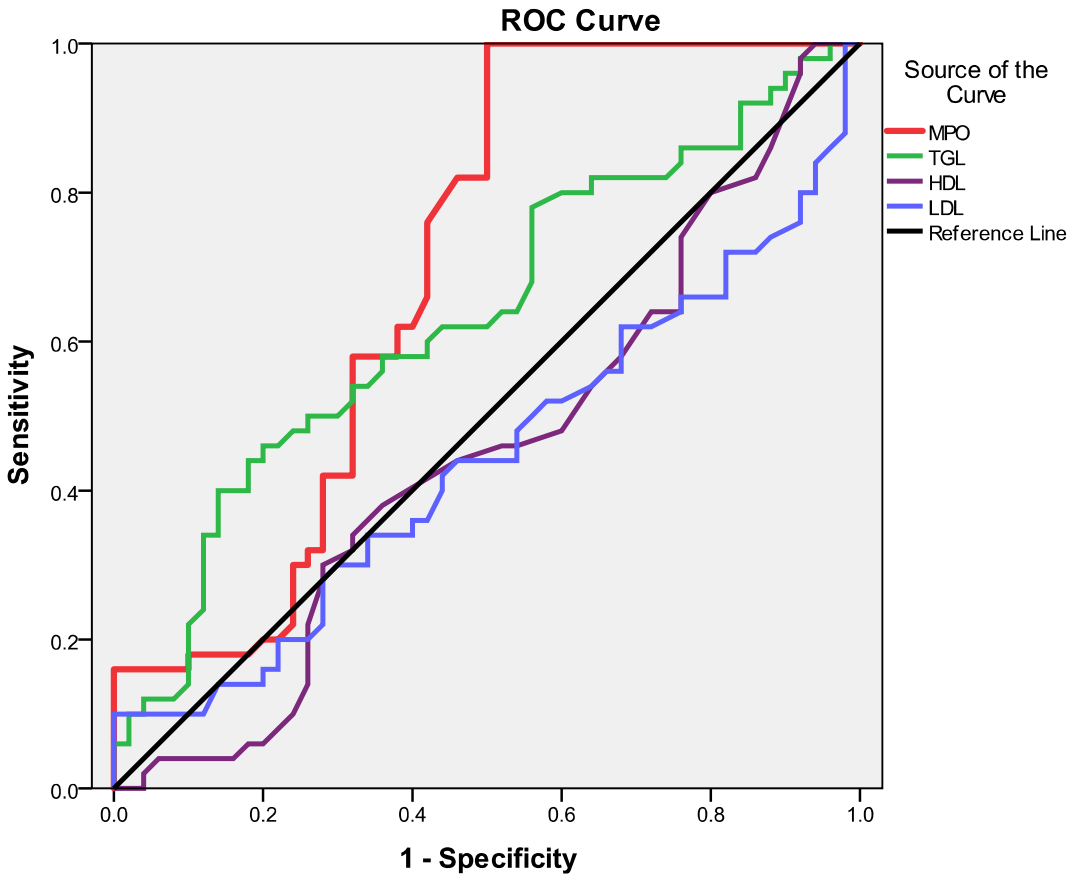


Table 6.8: Comparison of area under the curves for different parameters:

Variable	Area	Significance
Triglyceride	0.635	0.02*
HDL	0.455	0.436
LDL	0.442	0.319
MPO	0.696	0.001*

*p <0.05 – Statistically significant

DISCUSSION

Diabetes mellitus is characterised by chronic hyperglycemia due to relative insulin deficiency or insulin resistance or both. Chronic hyperglycemia is the primary etiological factor for the development of vascular complications of DM.

DM is associated with low-grade inflammation and amplified endothelial dysfunction. Persistent hyperglycemia in diabetes can aggravate inflammation and lead to increased production of reactive oxygen species from glucose auto-oxidation which can predispose to harmful consequences in diabetic patients. Endothelial dysfunction is a major contributing factor to the pathogenesis of diabetic vascular complications. ROS such as superoxide ions and hydrogen peroxide have emerged as important molecules in the pathogenesis of diabetic vascular complications, including endothelial dysfunction. Vascular NADPH oxidase, the non-leukocyte oxidase, has proven to be the major source of ROS in diabetic vasculature [73]. Increased levels of MPO activity contribute to initiation and accelerated progression of atherosclerosis in diabetes [85]. MPO can use high-glucose stimulated, vascular non-leukocyte derived hydrogen peroxide to induce diabetic endothelial dysfunction by reducing nitric oxide bioavailability [86].

In the present study, plasma MPO levels are significantly higher in the diabetic patients as compared to the non-diabetic controls ($p < 0.05$). This finding is consistent with the study by Wiersma *et al* [85] in which there was significant increase in the myeloperoxidase levels in diabetic patients. DM is considered to be a state of low grade and chronic inflammation. It has been also associated with accelerated atherosclerosis which predisposes these individuals to risk of cardiovascular disease. MPO-catalyzed reactions have been attributed to potentially pro-atherogenic biological activities throughout the evolution of cardiovascular disease, including initiation, propagation and acute complication phases of the stages of atherosclerosis [2]. This explains the increase in MPO activity in diabetic patients.

The mean fasting blood glucose concentrations is much higher in the plasma of the diabetic group (204.14 ± 75.64mg/dL). This increased glucotoxicitycan contribute to oxidative stress and increased inflammation which consequently results in increase in MPO activity in these patients. MPO can use high glucose stimulated hydrogen peroxide to amplify the toxic effects of increased glucose to the vascular wall [86]. MPO activity did not significantly differ with gender in both control group and diabetic group as depicted in Figures 6.9 & 6.10.

Plasma MPO activity in both the groups is found to increase with an increase in body mass index. MPO activity of > 400 U/L is more in the controls and cases with body mass index of ≥ 30 kg/m^2 as evidenced in Figures 6.7 & 6.8. Obesity was found to contribute to inflammation by up-regulating genetic elements involved in the inflammatory response. These genes control the expression of leucocytes called macrophages that play a key role in inflammation. As the concentration of macrophages in the fat tissue increases, the release of inflammatory by-products such as interleukins and others also increase.MPO is one of the inflammatory mediators which has been found to be increase in obese pre-pubertal children and served as a marker for cardiovascular disease in these children [87].

There is significant difference in the serum triglyceride levels in the two study groups (p value 0.02). DM is associated with clustering of lipid and lipoprotein abnormalities. One important dyslipidemia in these individuals which is an independent risk factor for cardiovascular complications is elevated triglyceride levels. This has been attributed to the increased hepatic secretion of triglyceride rich VLDL and impaired clearance of VLDL from the circulation of diabetic patients [88]. There is no significant difference between the two groups in relation to other parameters of lipid profile.

Furthermore, Pearson's correlation analysis done to establish correlation between plasma MPO activity and parameters such as BMI, serum cholesterol, serum triglyceride, serum HDL, HbA_1c. It revealed a positive moderate correlation (r value of 0.4) between serum triglyceride levels and plasma MPO activity in diabetic patients. Although elevated LDL cholesterol is well established as a major predictor of coronary heart disease risk and has been the primary target for lipid-lowering strategies, evidence suggests that an elevated triglyceride level is also an independent risk factor [89]. Hypertriglyceridemia as a result of insulin resistance is a consequence of enhanced breakdown of lipids in the cells ofthe adipose tissue.

This results in flux of free fatty acid to the liver and increased secretion of VLDL. The increased triglyceride from VLDL further activates cholesterol ester transfer protein, which results in triglyceride enrichment of LDL and HDL. The triglyceride content within these particles is hydrolysed by lipases, which leads to formation of small, dense LDL and HDL particles. Such modified HDL becomes less functional. Modified small and dense LDL particles are more susceptible to modifications by oxidation. These modifications in the lipoproteins contribute to the pathogenesis of atherosclerosis [89]. These modifications in the lipoproteins can lead to atherosclerotic changes in the vessel wall

which contributes to the vascular complications of diabetes mellitus. In our study, the correlation between serum triglyceride levels and plasma MPO activity shows that these patients are more prone to cardiovascular disease than the healthy controls.

There is a weak inverse correlation between HDL levels and MPO activity (r value of -0.1). MPO has been found to impair the functions of HDL because of its capacity to convert anti-inflammatory HDL to pro-inflammatory HDL, thereby influencing endothelial dysfunction [3]. However there was no much significant effect on the serum HDL concentration in the present study. It has been demonstrated that the endothelial-vasoprotective effects of HDL are impaired in type 2 diabetic patients. The loss in beneficial functions of HDL in diabetic patient could be explained by the observed lipid peroxidation and increased MPO activity in diabetic patients. MPO has been shown to modify HDL and its capacity of reverse cholesterol transport [3].

There is mild significant correlation between MPO activity and serum cholesterol levels in diabetic patients. Increase cholesterol in the circulation gets deposited in the walls of the arteries which leads to the formation of foam cells and initiates the process of atherosclerosis. The correlation between MPO activity and serum cholesterol levels shows the

efficiency of MPO as a risk indicator for predicting cardiovascular dysfunction in diabetic patients.

In this study, there is mild positive correlation between HbA1c levels and MPO activity in the diabetic patients (r value of 0.235). Large volume of data from various populations has established high HbA_1C level related with a rise in the occurrence of complications associated with DM. Glycated proteins lose their functional capacity and contribute to tissue damage and increased oxidative stress which contributes to vascular complications of diabetes mellitus [18].

The rise in MPO activity is irrespective of the duration of diabetes. Though increase in the duration of diabetes had been associated with increased glucotoxicity which contributes to vascular morbidity, in our study there was no significant effect of the duration of diabetes on MPO activity.ROC curve for MPO activity had an area under the curve of 0.696. When compared with ROC curves for serum triglycerides, serum HDL, serum LDL it was found that MPO activity followed by triglyceride levels had a larger area under the curve than serum HDL and serum LDL. This suggests the fact that plasma MPO activity and serum triglyceride levels should be given more emphasis in the clinical setting along with serum HDL and serum LDL levels. Increase in serum triglyceride levels are an independent cardiovascular morbidity and mortality predictor in diabetic patients [89].

CONCLUSION

DM is an inflammatory condition which is characterised by chronic hyperglycemia. The increased glucose levels leads to the development of vascular complications in DM. Several markers have been established to identify the vascular disturbances in these patients at an earlier stage. MPO has been concerned in the development of all phases of atherosclerotic development by promoting LDL oxidation, producing dysfunctional HDL and reducing nitric oxide bioavailability.

In the present study

- ✓ Plasma MPO activity was significantly higher in the diabetic patients when compared with non-diabetic controls.

- ✓ There is significantly good positive correlation between MPO activity and serum triglyceride levels.

- ✓ ROC analysis was performed. The areas under the curve for MPO activity and lipid profile parameters shows that MPO activity is a better significant marker followed by serum triglyceride levels.

Inflammatory response may have a dual role in DM, either it can have a causal relationship leading to insulin resistance or lead to the series of events which progresses to atherosclerosis ensuing in vascular complications.

In conclusion, inflammatory pathways play an essential role in the progress and evolution of vascular complications in DM. Modulation of inflammatory processes in diabetes by therapeutic intervention will have beneficial actions on patients with DM.

SUMMARY

Myeloperoxidase, a heme peroxidase is a lysosomal protein released from the azurophil granules of the neutrophils. It is found to be an integral part of the innate immune response, by catalysing the formation of numerous reactive oxygen species. The products derived from MPO-catalyzed reactions have been ascribed to several pro-atherogenic biological activities throughout the advancement of cardiovascular disease. It has been implicated in all stages of atherosclerotic process including initiation, propagation, and acute complication phases.

Diabetes mellitus, a multisystem disorder is a common and growing public health problem. The role of endothelial dysfunction in the causation of vascular disease in diabetes mellitus has gained increasing attention. In diabetes mellitus, there is also an accelerated rate of the atherosclerotic process which further contributes to the vascular complications. Cardiovascular disease is found to be more common in the diabetic population than non-diabetic individuals.

This study was conducted to estimate the plasma MPO activity in diabetic individuals and compare them with that of non-diabetic healthy controls. Further the study also sought to establish any correlation

between plasma MPO activity and lipid profile parameters and also with that of HbA_1c.

The study involved two groups of subjects namely, healthy non-diabetic controls and diabetic patients, 50 in each group. Plasma MPO activity was estimated in the study population spectrophotometrically using o-dianisidine as the substrate and the results were expressed in U/L. The lipid profile parameters such as serum total cholesterol, serum triglyceride, serum HDL and serum LDL were analysed in auto-analyser. It was found that plasma MPO activity was higher in the diabetic patients (317.17±247.73 U/L) when compared to healthy controls (188.41±142.73U/L). This difference was statistically significant. A positive correlation that was statistically significant was observed between plasma MPO activity and serum triglyceride levels in the diabetic patients.

The increase in MPO activity in diabetic patients could be attributed to the hyperglycemia induced oxidative stress, inflammation and also the increased propensity of the diabetic individuals to atherosclerotic process. MPO and associated inflammatory pathways signify striking targets for both predictive and beneficial involvement in the prevention of atherosclerotic cardiovascular disease.

SCOPE FOR FUTURE STUDY

The study needs to be carried out on larger population size along with more sensitive markers of inflammation to make sense of their exact role in the onset and progression of DM. The question whether MPO is a marker of an increased inflammatory state and leukocyte count in diabetic patients or whether MPO plays an independent role in diabetic associated accelerated atherosclerosis cannot be answered in this study.

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DATA COLLECTION TOOL

1. Patient's Name :
2. Patient's ID :
3. Age :
4. Sex :
5. Height :
6. Weight :
7. Patient's History :

7.1.	Diabetes	:	1.Yes	2.No
7.2.	Duration of Diabetes	:		
7.3.	Hypertension	:	1.Yes	2.No
7.4.	Recent history of Infection	:	1.Yes	2.No
7.5.	Recent history of hospitalization	:	1.Yes	2.No
7.6.	History of Myocardial Infarction	:	1.Yes	2.No
7.7.	History of Kidney Disease	:	1.Yes	2.No
7.8.	History of thyroid disorders	:	1.Yes	2.No
7.9.	Any known co-existing morbidity	:	1.Yes	2.No
7.10.	If yes, details	:		
7.11.	History of Drug intake	:		

Master chart for controls

S.No	Age in years	Gender	BMI Kg/m^2	Fasting blood sugar mg/dL	Serum Total Cholesterol mg/dL	Serum triglyceride levels mg/dL	Serum HDL Cholesterol mg/dL	Serum LDL Cholesterol mg/dL	Plasma MPO activity U/L
1	34	F	17.10	104	232	156	46	168	176.15
2	51	M	18.56	107	144	71	35	94	37.37
3	39	F	19.57	85	156	86	61	123	16.01
4	46	M	19.71	91	200	152	87	128	362.98
5	44	F	20.06	94	240	140	41	170	53.38
6	54	M	20.34	88	185	146	33	131	160.14
7	50	M	21.14	97	136	127	65	154	339.14
8	52	M	21.46	114	192	147	35	127	181.49
9	43	F	22.1	83	102	123	39	134	85.408
10	38	F	23	95	135	105	46	108	69.394
11	58	M	23.05	86	165	312	31	86	144.13
12	49	M	23.83	95	200	127	28	112	459.07
13	42	F	23.94	99	229	91	59	146	379.00
14	32	F	24	103	150	154	48	85	96.084
15	65	M	24.03	109	216	91	60	137	85.41
16	40	F	24.34	93	241	82	61	164	106.76
17	48	M	24.39	97	176	128	42	123	106.76
18	48	M	25.00	115	113	97	18	67	368.32
19	44	F	25.40	115	140	60	29	91	53.38
20	48	M	25.59	97	212	109	38	124	272.24
21	36	F	25.71	107	227	212	40	160	48.04
22	56	M	25.77	91	144	45	41	93	96.08
23	40	F	26.1	89	125	100	54	120	48.042
24	41	F	26.13	87	134	108	20	93	101.42
25	33	F	26.17	97	185	108	39	133	381.84
26	54	M	26.22	96	170	121	43	106	69.39
27	61	M	26.37	88	183	147	42	121	160.14
28	52	M	26.42	120	206	99	38	146	213.52
29	33	F	26.73	106	224	131	50	150	69.39
30	45	M	26.91	94	169	63	55	97	106.76
31	55	M	27.34	90	164	205	32	103	324.13
32	50	M	27.51	102	205	120	45	139	48.04
33	57	M	27.59	90	172	192	44	104	373.66
34	44	F	27.72	81	252	138	77	151	37.37

S.No	Age in years	Gender	BMI Kg/m^2	Fasting blood sugar mg/dL	Serum Total Cholesterol mg/dL	Serum triglyceride levels mg/dL	Serum HDL Cholesterol mg/dL	Serum LDL Cholesterol mg/dL	Plasma MPO activity U/L
35	65	M	27.78	95	223	213	44	161	517.79
36	61	M	27.83	92	178	54	63	103	101.42
37	50	M	28.60	102	234	160	40	125	213.52
38	52	M	29	90	132	132	55	98	102.42
39	60	M	29.09	96	195	80	45	136	37.37
40	34	F	30.12	87	155	180	33	145	365.83
41	44	F	30.13	103	200	273	45	113	416.36
42	40	F	30.86	100	173	95	45	118	416.36
43	42	F	31.10	85	212	168	31	155	145.58
44	63	M	31.20	98	174	127	48	107	53.38
45	45	F	32.4	87	142	98	60	115	53.38
46	57	M	33.32	115	162	41	42	106	373.66
47	54	M	33.67	102	230	109	56	152	213.52
48	52	M	34.25	96	200	92	33	148	266.90
49	57	M	35.84	103	195	169	45	124	96.08
50	48	M	36.11	84	181	135	44	115	416.36

Master chart for cases

S.No	Age	Gender	BMI kg/m^2	Fasting blood sugar mg/dL	Serum Total Cholesterol mg/dL	Serum triglceride levels mg/dL	Serum HDL cholesterol mg/dL	Serum LDL cholesterol mg/dL	Plasma MPO activity U/L	HbA_1c	Duration of DM
1	45	M	18.67	158	137	85	39	92	138.79	7.28	5
2	57	F	19.38	236	260	136	52	188	288.25	12.65	20
3	48	M	19.56	198	187	113	59	121	160.14	9.80	1
4	51	F	19.95	313	198	85	50	144	245.55	11.25	13
5	48	F	20.55	131	160	107	32	109	298.93	8.19	5
6	60	M	21.34	133	253	296	41	175	352.35	6.16	10
7	55	M	22.14	185	210	176	45	145	321.52	9.52	14
8	49	F	22.15	200	228	95	51	154	362.98	11.63	8
9	69	M	22.31	168	192	120	53	122	218.86	6.55	7
10	54	F	22.52	250	166	342	35	92	165.48	9.66	5
11	65	F	22.66	184	207	112	41	149	585.25	6.50	17
12	59	M	23.05	233	200	163	35	135	133.45	6.93	1
13	40	M	23.07	216	155	205	32	70	368.32	6.90	2
14	45	M	23.26	345	137	162	39	80	234.87	6.68	3
15	53	M	24.06	195	191	182	31	140	165.48	13.69	10
16	79	M	24.22	136	140	200	45	100	197.51	6.65	15
17	69	M	24.39	195	144	62	73	99	133.45	7.59	25
18	40	F	24.59	225	180	150	50	172	293.59	9.20	1
19	58	F	24.88	184	197	138	46	144	138.79	5.90	10
20	54	M	25.71	168	198	203	31	147	128.11	11.83	3
21	44	M	25.82	154	198	192	31	126	122.77	8.40	2
22	37	M	25.86	165	130	118	37	81	133.45	6.94	8
23	43	M	26.30	135	220	109	37	79	170.82	6.10	2
24	55	M	27.06	145	244	572	31	130	1110.30	11.90	1
25	54	F	27.18	123	190	64	55	111	160.14	7.80	15
26	42	M	27.25	345	182	143	44	127	352.31	9.70	1
27	50	F	27.41	142	164	94	54	105	544.45	6.40	5
28	64	M	27.58	123	176	72	54	115	341.63	7.08	3
29	72	F	27.97	225	187	123	45	122	176.15	7.88	21
30	40	F	28.13	362	180	200	65	85	128.11	6.80	3
31	58	F	28.69	346	195	219	41	127	245.55	6.82	3
32	68	M	29.00	235	198	159	47	113	160.14	7.40	5
33	32	F	29.00	154	250	185	30	120	520.45	8.45	4
34	44	M	29.37	320	180	197	35	125	544.48	6.51	3
35	32	M	29.41	154	269	120	37	189	128.11	6.50	1
36	31	M	29.43	140	165	46	37	90	1035.57	8.41	4

S.No	Age	Gender	BMI kg/m^2	Fasting blood sugar mg/dL	Serum Total Cholesterol mg/dL	Serum triglceride levels mg/dL	Serum HDL cholesterol mg/dL	Serum LDL cholesterol mg/dL	Plasma MPO activity U/L	HbA_1c	Duration of DM
37	40	F	29.55	154	200	172	29	76	325.62	6.90	1
38	50	M	29.64	256	178	110	52	185	165.48	6.39	8
39	43	F	30.48	123	180	170	36	100	402.25	6.50	1
40	52	M	30.49	348	189	291	40	111	261.56	6.20	3
41	55	F	30.82	154	271	400	50	139	1225.65	11.10	6
42	65	M	32.03	126	150	181	33	94	176.15	6.56	10
43	38	M	32.28	186	148	191	39	93	314.94	6.10	2
44	63	M	32.47	143	150	128	49	88	752.66	7.10	9
45	38	M	32.83	150	154	191	46	83	165.48	6.46	1
46	63	M	33.15	119	165	83	40	106	208.18	7.50	6
47	48	F	34.02	205	198	154	55	154	340.62	6.45	13
48	36	M	35.13	210	221	129	42	147	224.20	6.60	2
49	45	M	35.38	354	175	146	48	135	265.32	8.24	11
50	65	M	41.01	365	238	117	31	86	224.20	6.32	25